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Early Detection and Identification of the Main Fungal Pathogens for Resistance Evaluation of New Genotypes of Forest Trees

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Abstract: Growing importance of forest plantations increases the demand for phytopathogen resistant forest trees. This study describes an effective method for the early detection and identification of the main fungal phytopathogens in planting material of silver birch (*Betula pendula*) and downy birch (*B. pubescens*) based on the estimation of size of the internal transcribed spacers (ITS1 and ITS2) in the 18S-5.8S-28S rDNA gene cluster, which are species-specific for most micromycetes. Electrophoretic assay of the ITS1 and ITS2 loci allowed us to identify the predominant phytopathogenic fungal species in downy and silver birch *in planta*. This new molecular genetic method can be used to screen birch and other forest trees for different fungal pathogens in evaluation of disease resistance. This information can be useful in breeding of new genotypes of forest trees including transgenic clones with modified wood composition.

Keywords: *Betula*; birch; fungal phytopathogens; ITS

1. Introduction

Fungal diseases are a serious problem in forestry and sometimes they can be the cause of epiphytotics, leading to the death of forests, for example, American chestnut, butternut, and American elm [1]. This is particularly relevant for forest plantations, which have less diversity and stability than natural forests and, therefore, are more susceptible to diseases. Modeling indicated that short rotations in forest plantations accelerated both the virulence evolution in root-rot pathogenic

45 fungi and the development of the epidemics [2]. Moreover, global climate change may also promote
46 the forest pathogens. It has been shown that expected changes in temperature and precipitation will
47 favor the spread of beech bark disease in the forests of North America [3]. Thus, special attention is
48 needed to assess the disease resistance of new genotypes of forest trees, including transgenic lines.
49 Lignin manipulation is one of the main objectives in forest biotechnology. Its content in wood directly
50 correlates with the efficiency of the pulping process and affects also waste management. However,
51 lignin plays an important role in plant defense against pests and other phytopathogens [4]. Thus, a
52 change in the composition and/or content of lignin can reduce plant resistance to phytopathogens. In
53 general, in addition to the biotechnologically generated desirable traits (intended effects), the
54 appearance of unintended effects is possible that can negatively affect an agronomic performance [5].
55 Detection of such effects can be done by comparing transgenic genotypes with related conventional
56 counterparts [6]. In case of testing whether transgenic genotypes that have lower lignin content are
57 less resistant to phytopathogens it would mean detection of these phytopathogens in transformed
58 and untransformed clones.

59 Birch species (*Betula*L.) are among the most widespread forest trees that have great importance
60 in forestry, forest formation and soil improvement. They also have important ecological role as
61 pioneer species after clearcuts and forest fires [7]. They are fast growing species and provide high
62 quality timber for industrial purposes. Downy birch (*Betula pubescens* Ehrh.) and silver birch (*B.*
63 *pendula* Roth) are commercially important forest species in Europe [8]. Their natural area includes
64 North Africa, Western Asia, and Central Asia, as well as entire Europe and Northern Eurasia,
65 excluding the Iberian Peninsula. In Northern Europe these species are the most important deciduous
66 trees in plantation forestry [9]. Intensively managed forest plantations are characterized by a limited
67 number of clones of the same species, which increases the risk of pathogen attacks. In this regard,
68 methods for the diagnosis and identification of various phytopathogens based on DNA analysis have
69 great potential value [10]. Molecular diagnostic methods have been developed for detection of various
70 pathogens in oak [11], plane trees [12], pines [13] and other forest species, but not in birch. Moreover,
71 these methods were designed to identify pathogens of only one particular species or genus in a single
72 analysis.

73 Traditional method of phytopathogen detection is based on visual inspection of disease
74 symptoms that is often unreliable, performed in the late stages of the disease and requires qualified
75 personal [14], especially for tree species. Molecular diagnostics based on detection of pathogen DNA
76 using PCR methods allow assessing the resistance of a new genotype quickly, with high accuracy
77 and at the early stages of the disease development [15]. For several reasons, ribosomal DNA (rDNA)
78 loci encoding 5S, 5.8S, 18S and 28S ribosomal RNAs (rRNAs) are widely used marker regions for the
79 detection and identification of micromycetes [16]. The rDNA loci encoding 5.8S, 18S and 28S rRNAs
80 form a cluster of the 18S-5.8S-28S loci with two internal transcribed spacers (ITS1 and ITS2) between
81 18S and 5.8S and 5.8S and 28S loci, respectively. There are at least 50 copies of this cluster per genome,
82 and this multiplicity enhances the sensitivity of the PCR analysis, *i.e.* the probability of pathogen
83 detection at low concentration in plant tissue. The ITS loci are relatively conserved within a species
84 [16], but highly divergent between species, which facilitates taxonomic identification the pathogen
85 causing infection. These loci are well studied and their nucleotide sequences are well-represented in
86 sequence databases, such as NCBI GenBank (https://www.ncbi.nlm.nih.gov), DNA DataBank of
87 Japan (DDBJ, NIG) (http://www.ddbj.nig.ac.jp), European Molecular Biology Laboratory (EMBL,
88 EBI) (http://www.embl.de), Barcode of Life Data System (BOLD) (http://www.boldsystems.org),and
89 DOE JGI Fungi Portal (https://genome.jgi.doe.gov/programs/fungi/index.jsf), which are very
90 important for pathogen identification. Large scale molecular genetic studies of different fungi have
91 revealed conserved rDNA regions and allowed the development of sets of universal primers for PCR
92 amplification of ribosomal genes and intergenic spacers across different species [17]. DNA-based
93 methods have been proposed for the identification of fungal species based on electrophoretic assay
94 of the PCR amplified marker regions without preliminary sequencing of samples, including the
95 identification of micromycetes [18]. The ITS region has been relatively recently suggested as a
96 universal marker for DNA barcoding of fungi [18]. However, most of the proposed protocols are not

universally applicable and have limitations in different phytopathological assays. For example, the use of an intergenic spacer (IGS) located between tandemly repeated copies of the rDNA gene clusters as a genetic marker may be limited for studying pathogenic basidiomycetes because of the high sequence variation in this region within species, and the challenges of amplifying DNA regions larger than 3 kilobase pairs (Kbp), particularly from decayed tissues [19]. Application of an SSCP (single-strand conformation polymorphism) analysis does not directly generate nucleotide sequence data, which reduces their compatibility with nucleotide sequence databases [20]. Moreover, the likelihood of methodological mistakes and artifacts becomes greater when complex procedures are required for sample preparation and electrophoretic mobility analysis [21]. Finally, the sequencing of DNA markers involves a relatively high analytical cost and specialist laboratory equipment.

In the present study, we developed the ITS1 and ITS2 genetic markers that can be used without sequencing. Their species-specific variation in size makes them highly informative and sufficient for identification of the main pathogenic species of birches using gel electrophoresis following PCR amplification. It is very important that there is almost no intraspecific size variation of the ITS markers in micromycetes that could be similar to the interspecific size variation, which almost excludes false positive results. Amplicon size analysis was carried out by denaturing polyacrylamide gel electrophoresis, which allowed for species identification using also both application of standard DNA samples and information about amplicon sizes from nucleotide sequence databases.

2. Materials and Methods

2.1. Plant Material

Samples from silver and downy birch planting with different infection symptoms were collected during 2017 in fields and greenhouses at the Korenevskaya Experimental Forest Enterprise of the Forest Research Institute of the National Academy of Sciences of Belarus (Belarus), and at the forest enterprises of Gomel Region (Belarus) and Moscow Region (Russia).

2.2. Phytopathological Analysis

During phytopathological assay of the birch planting material, the main diseases that caused the highest losses of yield during commercial cultivation were determined. Determination of disease type was carried out based on the symptoms defined by a generally accepted system of phytopathological assays (http://www.forestpathology.org/index.html).

2.3. Species-specific Molecular Genetic Identification of Phytopathogens

Specific phytopathogenic micromycetes were identified using molecular genetic methods for fungal identification *in planta* [22]. For pathogen diagnosis, samples of plant tissue were collected at the initial infection stage, which simplified the diagnosis by minimizing the content of saprotrophic microflora. All the plant samples (e.g., cuttings of leaf discs, stem or root fragments) were fixed in sterile polypropylene tubes with 70% ethanol and store at −18 °C. During sample preparation, analyzed fragments of plant material were removed from the tubes, washed thoroughly with running water, and pieces that exhibited a particular infection type were taken for further analysis. They were washed thoroughly with distilled water and cut with a razor blade into 3-8 mm pieces under sterile conditions, such that a junction between healthy and infected tissues was located in the middle of each piece. Samples were then placed in Eppendorf centrifuge tubes for subsequent DNA isolation.

2. 4. DNA Isolation and PCR amplification

Total DNA was extracted from the samples according to a modified cetyltrimethyl ammonium bromide (CTAB) protocol [23]. PCR was carried out using the 2×DreamTaq™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with combinations of ITS1-ITS2 (amplifying partial 18S rRNA, ITS1, and partial 5.8S rRNA loci) or ITS3-ITS4 (amplifying partial 5.8S rRNA, ITS2, and partial 26S rRNA loci) PCR primer pairs for amplification of the fungal rDNA species-specific genetic markers[16]. Forward primers were labeled with a fluorescent dye. Primer sequences are shown in Table 1. The amplification reaction mixture (25 µL) contained 1 µL (0.5-50 ng) of DNA template, 12.5 µL of 2× DreamTaq™ Green PCR Master Mix, 1 µL of 5 µM Dye-labelled (e.g., with FAM-dye) forward primer, 1 µL of 5 µM reverse primer and 9.5 µL nuclease-free water. DNA reaction mixtures were amplified in a PCR thermocycler (TProfessional Basic Thermocycle)

148 (Biometra GmbH, Göttingen, Germany) by algorithm: 1 cycle at 95°C for 3 min, followed by 35 cycles
149 of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. The reaction was ended with a final extension at 72°C
150 for 4 min before holding the sample at 4°C for analysis.
151
152 **Table 1.** Primer sequences used for the PCR amplification of the fungal ITS1 and ITS2 loci.

Locus	Primer	Primer sequence (5′–3′)
ITS1	ITS1	FAM-TCCGTAGGTGAACCTGCGG
	ITS2	GCTGCGTTCTTCATCGATGC
ITS2	ITS3	FAM-GCATCGATGAAGAACGCAGC
	ITS4	TCCTCCGCTTATTGATATGC

153
154 *2. 5. Gel Electrophoresis*
155 For high resolution gel electrophoresis and amplicon fragment analysis the PCR products were
156 diluted to 1 ng/μl in deionized water, and 1 μl of diluted PCR product was mixed with 18 μl of
157 formamide and 1 μl of GeneScan™ 500 LIZ™ dye Size Standard (Thermo Fisher Scientific, Waltham,
158 Massachusetts, USA) used as internal molecular weight markers. The mix was heated to 95°C for 5
159 min to denature the products into single DNA strands and then cooled immediately on ice for 2 min.
160 The denatured PCR products were then loaded into ABI Prism 310 Genetic Analyzer (Thermo Fisher
161 Scientific, Waltham, Massachusetts, USA) and electrophoretically separated in POP-4 polymer
162 according to the manufacturer's manual. The fragment calls and analysis were performed using the
163 GeneMapper v. 4.0 software (Thermo Fisher Scientific, Massachusetts, USA). In parallel, all
164 alternatively sized amplicon variants were sequenced. Initial species identification, based on the
165 amplicon sequences, was carried out using an on-line BLAST tool
166 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequent species identification was based on the
167 determination of species-specific fragments with unique size that represented particular fungal
168 species in the ABI-generated electrophoregram with multiple peaks representing amplicon sequences
169 in the PCR amplified sample (Figure 2).

170 **3. Results and Discussion**

171 Traditional phytopathological assay determined list of the fungal infection in birch planting
172 material as leaf (leaf spots, powdery mildew and rust leaves), shoot (necrosis and cancer pathologies),
173 root, and vascular system (rot and wilting) diseases (Figure 1; [24]). The various leaf diseases were
174 the most common with a predominance of powdery mildew (23.2%). In addition, for precise genetic
175 identification of fungal pathogens we used molecular genetic markers representing nucleotide
176 sequences of the ITS1 and ITS2 regions. They indicated that multiple species of micromycetes were
177 present in more than 80% of the infected plant tissue samples, although typically one or several fungal
178 species were predominated. Dominant phytopathogenic micromycetes species were detected both in
179 association with other fungal species, as well as alone, suggesting key role for these fungi in
180 pathogenesis. The presence of other minor micromycetes showed no particular pattern in the
181 different plants samples, either alone or as part of associations with other microbes, and, after species
182 identification, they appeared to represent a group of secondary pathogens and saprophytic fungi.
183 Table 2 presents a list of the main 12 phytopathogenic fungal species based on the ITS1 and ITS2
184 markers.
185

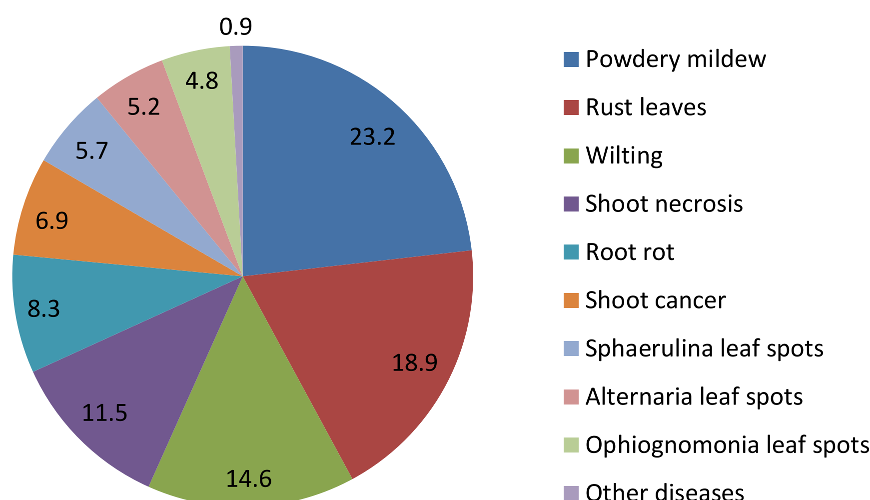

Figure 1. Infectious diseases identified in the downy birch and silver birch planting based on visual phytopathological inspection (%).

Table 2. The occurrence of the main phytopathogenic fungal species and diseases in the silver and downy birch planting.

Phytopathogen	Disease type	Occurrence, %
Phyllactinia guttata	Powdery mildew	13.2
Microsphaera betulae	Powdery mildew	10.0
Melampsoridium betulinum	Rust leaves	18.9
Fusarium avenaceum	Wilting	13.2
Nectria sp.	Shoot necrosis	4.1
Melanconium bicolor	Shoot necrosis	3.7
Phytophthora cactorum	Shoot necrosis	3.5
Pythium sp.	Root rot	7.6
Botryosphaeria dothidea	Shoot cancer	6.2
Ophiognomonia intermedia	*Ophiognomonia* leaf spots	4.8
Sphaerulina betulae	*Sphaerulina* leaf spots	5.7
Alternaria alternata	*Alternaria* leaf spots	4.9
Other species	Other diseases	0.9

The analysis showed that the causative agents of powdery mildew were two pathogens, *Phyllactinia guttata* and *Microsphaera betulae*, mainly the first one. *Melampsoridium betulinum* was the most common pathogen (18.9%).This fungus causes the birch rust, which is harmful in nurseries and also decreases seedling growth during the next spring after planting [25]. The disease was the most severe in downy birch [26]. There were clear genetic differences in susceptibility to rust among birch clones [27], and an effective diagnostic method for resistance to this pathogen will be useful in breeding. Leaf spots on birch are caused by a number of fungi [28]. In our study, the pathogens were *Ophiognomonia intermedia*, *Sphaerulina betulae* and *Alternaria alternate*, in approximately equal proportions. The analysis of the amplicon nucleotide sequences amplified by the ITS1 and ITS4 primer pair, which included the rDNA region representing partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 26S rRNA loci, showed that all revealed phytopathogens possessed a species-specific unique nucleotide sequence corresponding to the marker locus. Sizes of diagnostic loci in the same pathogen were identical for samples from different geographic regions. The amplicon size variation

was mainly due to polymorphism in the ITS1 and ITS2 loci (Table 3). The 5.8S rRNA gene and partial sequences of the 18Sand 26SrRNA genes varied only in a few cases. In general, the main interspecies differences were due to nucleotide substitutions [29].

Table 3. List of the main phytopathogenic fungal species identified in the silver birch and downy birch planting and sizes of their species-specific diagnostic ITS amplicons obtained using the ITS1-ITS2 and ITS3-ITS4primer pair combinations.

Phytopathogenic species	ITS1-ITS2, bp	ITS3-ITS4, bp
Sphaerulina betula Quaedvl., Verkley ex Crous	225	231
Ophiognomonia intermedia (Rehm) Sogonov	268	351
Alternaria alternata (Fr.) Keissl.	244	346
Phyllactinia guttata (Wallr.) Lev.	314	364
Botryosphaeria dothidea (Moug. ex Fr.) Ces. & DeNot.	259	344
Microsphaera betulae Magn.	298	362
Melampsoridium betulinum (Pers.) Kleb.	328	406
Pythium sp.	298	633
Phytophthora cactorum (Leb. and Cohn) Schroeter	295	602
Fusarium avenaceum (Fr.) Sacc.	233	355
Melanconium bicolor Nees.	270	349
Nectria sp.	217	348

Typical computer-generated capillary gel electrophoregrams derived for infected birch samples are presented in Figure 2. In the absence of fungal infection, with only the genomic DNA of silver or downy birch present as a template, only a single electrophoretic peak, corresponding to an amplicon DNA fragment of the host plant, should be present because the birch ITS regions have similar annealing sites for the ITS1, ITS2, ITS3 and ITS4 primers. Thus, it is either a 299 bp long fragment size when the ITS1-ITS2 primer pair is used (Figure 2), a 411 bp long fragment size when the ITS3-ITS4 primer pair is used. These fragments can be used as an additional internal control for the PCR reaction, and their absence may indicate a PCR or DNA isolation failure or other technical errors in the protocol. If a single or multiple pathogens are present, DNA fragments of more than one size should be amplified. One of them should correspond to the host DNA, while others would indicate a phytopathogenic or saprophytic infection. Species identification of phytopathogens is based on the amplicon sizes (Table 3). To improve the resolution of the method, the electrophoretic fragment analysis can be performed with PCR products amplified by both primer pairs - ITS1-ITS2 and ITS3-ITS4. It is also possible to multiplex the amplicon analysis using primers labeled by spectrally different fluorescent dyes.

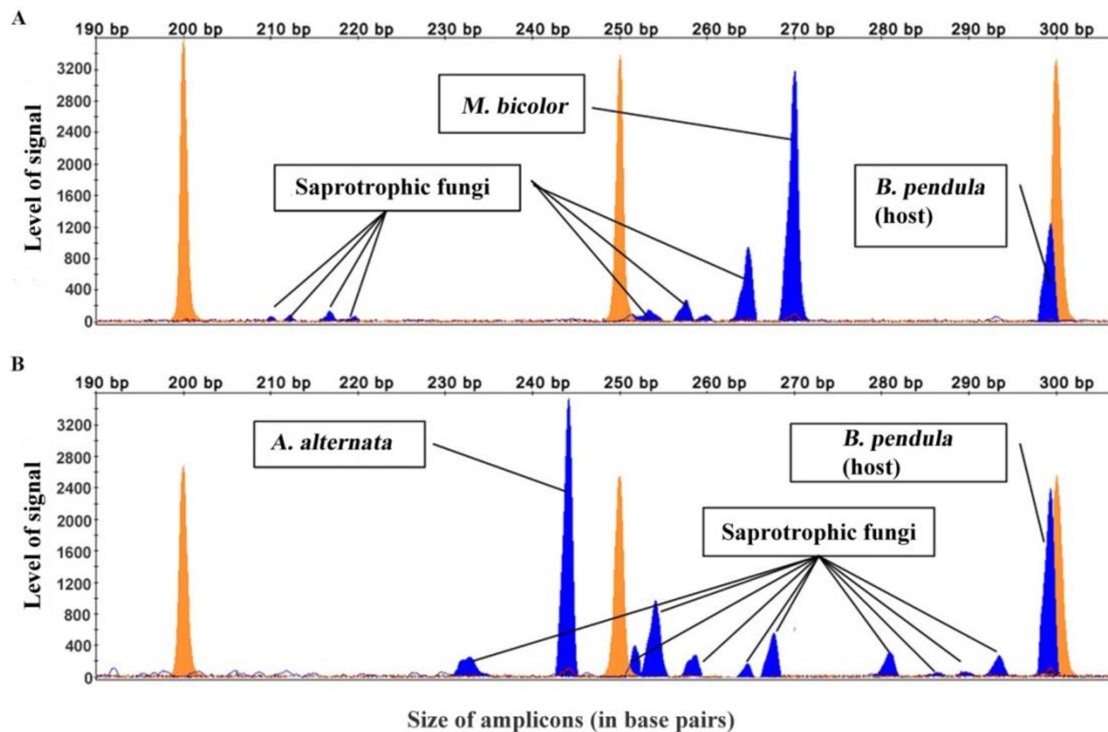

Figure 2. Example of two computer-generated electrophoregrams with multiple peaks representing species-specific fungal and host plant (*B. pendula*) amplicon DNA fragments from the two PCR-amplified samples of DNA isolated from infected silver birch leaf (A) and shoot (B)tissues, amplified using the ITS1-ITS2 primer pair combination and separated in the capillary gel electrophoresis using an ABI Prism 310 Genetic Analyzer. Brown peaks represent DNA fragments of the GeneScan™ 500 LIZ™ dye Size Standard.

The ITS regions of the rDNA were used for identification of fungal pathogen in forest trees [30], including resistance evaluation [31, 32], but only by sequencing DNA from pure microbial cultures. Alternatively, the 16S rRNA terminal restriction fragment length polymorphism (T-RFLP) method was used for profiling bacterial communities [33], but it required restriction enzyme treatment. We combined these two techniques and developed a method that allows fast and efficient detection and identification of fungal phytopathogens in plant samples without using pure cultures. We confirmed that the nucleotide structure of pathogen diagnostic loci, was conservative regardless of the geographic origin of the samples, and, therefore, the size of the amplified diagnostic loci can be reliably used for fungal species identification. This method allows studying mycobiomes of different plants by comparing their species compositions. We plan to use this method also for evaluation of disease resistance of transgenic aspen and birch clones with a modified wood composition [34].

5. Conclusions

We proposed a relatively simple method for molecular genetic detection and identification of phytopathogens and demonstrated its efficiency on the main species of phytopathogenic micromycetes. The method is based on the PCR fragment analysis of the ITS1 and ITS2 loci, which allows the identification of micromycetes without the need to sequence the amplicons. The proposed molecular genetic method is faster (processing time is approximately 4-5 hours), does not require designing species-specific PCR primers and less expensive than direct sequencing. The obtained results are more reliable that those that are based on species-specific PCR because cross-amplification is not problem for this method. It is also applicable for early assessing of disease resistance in new genotypes of forest trees developed for short-rotation plantations including transgenic clones. Moreover, this analysis allows the detection and identification of not only distinct species, but also their associations, thereby enabling metagenomic analyses.

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271 **References**

272 1. Schlarbaum, S.E.; Hebard, F.; Spaine, P.C.; Kamalay, J.C. Three American tragedies: chestnut blight,
273 butternut canker, and Dutch elm disease. Proceedings of the Exotic Pests of Eastern Forests Conference,
274 Nashville, TN, USA, 8-10 April 1997; Britton, K.O.; US Forest Service and Tennessee Exotic Pest Plant
275 Council, USA, 1997, pp. 45–54.
276 2. Soularue, J.-P.; Robin, C.; Desprez-Loustau, M.-L.; Dutech, C. Short rotations in forest plantations accelerate
277 virulence evolution in root-rot pathogenic fungi. *Forests***2017**, *8*, 205. DOI: https://doi.org/10.3390/f8060205
278 3. Stephanson, C.A.; Ribarik Coe, N. Impacts of beech bark disease and climate change on American beech.
279 *Forests***2017**, *8*, 155. DOI: https://doi.org/10.3390/f8050155
280 4. Liu, Q.; Luo, L.; Zheng, L. Lignins: Biosynthesis and biological functions in plants. *Int. J. Mol. Sci*. **2018**, *19*,
281 335. DOI: https://doi.org/10.3390/ijms19020335
282 5. Rischer, H.; Oksman-Caldentey, K.-M. Unintended effects in genetically modified crops: revealed by
283 metabolomics? *Trends Biotechnol*.**2006**, *24*, 102–104. DOI: 10.1016/j.tibtech.2006.01.009
284 6. Ladics, G.S.; Bartholomaeus, A.; Bregitzer, P.; Doerrer, N.G.; Gray, A.; Holzhauser, T.; Jordan, M.; Keese,
285 P.; Kok, E.; Macdonald, P.; Parrott, W.; Privalle, L.; Raybould, A.; Rhee, S.Y.; Rice, E.; Romeis, J.; Vaughn,
286 J.; Wal, J.-M.; Glenn, K. Genetic basis and detection of unintended effects in genetically modified crop
287 plants. *Transgenic Res*. **2015**, *24*, 587–603. DOI: https://doi.org/10.1007/s11248-015-9867-7
288 7. Den Herder, M.; Kouki, J.; Ruusila, V. The effects of timber harvest, forest fire, and herbivores on
289 regeneration of deciduous trees in boreal pine-dominated forests.*Can. J. For. Res*. **2009**, *39*, 712–722. DOI:
290 https://doi.org/10.1139/X08-208
291 8. Hynynen, J.; Niemiströ, P.; Viherä-Aarnio, A.; Brunner, A.; Hein S.; Velling, P. Silviculture of birch
292 (*Betulapendula* Roth and *Betulapubescens*Ehrh.) in northern Europe. *Forestry***2010**, *83*, 103–119. DOI:
293 https://doi.org/10.1093/forestry/cpp035
294 9. West, P.W. Choosing the species and site. In *Growing Forest Plantations*; West, P.W., Ed.; Springer
295 International Publishing: Basel, Switzerland, 2014; pp. 45-54. ISBN 978-3-319-01826-3
296 10. Shcherbakova L.A. Advanced methods of plant pathogen diagnostics. In *Comprehensive and Molecular*
297 *Phytopathology*, 1st ed.; Dyakov, Yu. T., Dzhavakhiya, V., Korpela, T., Eds.; Elsevier: Amsterdam, The
298 Netherlands, 2007; pp. 75–116. ISBN 978-0-444-52132-3
299 11. Bilodeau, G.J.; Pelletier, G.; Pelletier, F.; Levesque, C.A.; Hamelin, R.C. Multiplex real-time polymerase
300 chain reaction (PCR) for detection of *Phytophthoraramorum*, the causal agent of sudden oak death. *Can. J.*
301 *Plant Pathol*.**2009**, *31*, 195–210. DOI: https://doi.org/10.1080/07060660909507593
302 12. Pilotti, M.; Lumia, V.; Di Lernia, G.; Brunetti, A. Development of real-time PCR for in wood-detection of
303 *Ceratocystisplatani*, the agent of canker stain of *Platanus* spp. *Eur. J. Plant Pathol*. **2012**, *134*, 61–79. DOI:
304 https://doi.org/10.1007/s10658-012-0022-5
305 13. Luchi, N.; Oliveira Longa, C.M.; Danti, R.; Capretti P.; Maresi G. *Diplodiasapinea*: the main fungal species
306 involved in the colonization of pine shoots in Italy. *For.Pathol*.**2014**, *44*, 372–381. DOI:
307 https://doi.org/10.1111/efp.12109
308 14. Liu, B.; Zhang, Y.; He, D.; Li, Y. Identification of apple leaf diseases based on deep convolutional neural
309 networks. *Symmetry***2018**, *10*, 11. DOI: https://doi.org/10.3390/sym10010011
310 15. Bakonyi, J.; Nagy, Z. Á.; Rsek, T. É. PCR-based DNA markers for identifying hybrids within
311 *Phytophthoraalni*. *J. Phytopathol*. **2006**, *154*, 168–177. DOI: https://doi.org/10.1111/j.1439-0434.2006.01079.x
312 16. White, T.J.; Bruns T.D.; Lee S.B.; Taylor J.W. Amplification and direct sequencing of fungal ribosomal RNA
313 genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications; Innis, M.A., Gelfand,

D.H., Sninsky, J.J., White T.J., Eds.; Academic Press: San Diego, CA, USA, 1990; pp. 315-322. ISBN 978-0123721815

17. Elder, J.F. Jr.; Turner, B.J. Concerted evolution of repetitive DNA sequences in eukaryotes. *Q. Rev. Biol*. **1995**, *70*, 297–320. DOI: https://doi.org/10.1086/419073
18. Schoch,C.L.; Seifert,K.A.; Huhndorf,S.; Robert,V.; Spouge,J.L.; Levesque,C.A.; Chen,W. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proc. Natl. Acad. Sci. U. S. A.* **2012**,*109*, 6241–6246.doi:https://doi.org/10.1073/pnas.1117018109
19. Arteau, M.; Labrie, S.; Roy, D. Terminal-restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis profiling of fungal communities in Camembert cheese. *Int. Dairy J*. **2010**, *20*, 545–554. DOI: https://doi.org/10.1016/j.idairyj.2010.02.006
20. Callon, C.; Delbes, C.; Duthoit, F.; Montel, M.C. Application of SSCP-PCR fingerprinting to profile the yeast community in raw milk Salers cheeses. *Syst. Appl. Microbiol*. **2006**, *29*, 172–180. DOI: https://doi.org/10.1016/j.syapm.2005.07.008
21. Gori, K.; Ryssel, M.; Arneborg, N.; Jespersen, L. Isolation and identification of the microbiota of Danish farmhouse and industrially produced surface-ripened cheeses. *Microb. Ecol*. **2013**, *65*, 602–615. DOI: 10.1007/s00248-012-0138-3
22. Padutov, V.E.; Baranov, O.Yu.; Voropaev, E.V. *Molecular genetic analysis methods*; Unipol: Minsk, Belarus, 2007; 176 p., ISBN 978-985-6768-12-8
23. Rogers, S.O.; Bendich, A.J. Extraction of total cellular DNA from plants, algae and fungi. In *Plant Molecular Biology Manual*; Gelvin S.B., Schilperoort R.A., Eds.; Springer: Netherlands, 1994; pp. 1-8. ISBN 978-94-011-7654-5
24. Panteleev, S.V. Molecular genetic diagnosis and identification of fungal pathogens of planting material of tree species in forest nurseries of Belarus. Ph.D. Thesis, Forest Institute, Gomel, Republic of Belarus, 2013 (in Russian).
25. Poteri, M. *Screening of birch, Betula spp., for rust resistance to Melampsoridiumbetulinum*; Research Papers; Finnish Forest Research Institute: Helsinki, Finland, 1998; Volume 689, p. 44.
26. Jalkanen, R. Synthesis and new observations on needle pathogens of larch in Northern Finland. *Forests***2016**, *7*, 25. DOI: https://doi.org/10.3390/f7010025
27. Organisation for Economic Co-operation and Development (OECD). Consensus Document on the Biology of European White Birch (Betulapendula Roth). In *Series on Harmonisation of Regulatory Oversight in Biotechnology*, No. 28; OECD Environment Directorate: Paris, France, 2003; 46 p.
28. Phillips, D.H.; Burdekin, D.A. Diseases of ash (*Fraxinus* spp.), birch (*Betula* spp.) and alder (*Alnus* spp.). In *Diseases of Forest and Ornamental Trees*; Phillips, D.H., Burdekin, D.A., Eds.; Palgrave Macmillan; London, UK, 1992; pp. 284–298. ISBN 978-1-349-10955-5
29. Baranov, O. Yu.; Panteleev, S.V. Molecular genetic assay of rDNA loci of main forest tree species pathogens in Belarus. *Challenge of Forestry* **2012**, *72*, 220–223 (in Russian).
30. Broders, K.; Munck, I.; Wyka, S.; Iriarte, G.; Beaudoin, E. Characterization of fungal pathogens associated with white pine needle damage (WPND) in Northeastern North America. *Forests***2015**, *6*, 4088–4104. DOI: https://doi.org/10.3390/f6114088
31. Hauptman, T.; Ogris, N.; de Groot, M.; Piškur, B.; Jurc, D. Individual resistance of *Fraxinus angustifolia* clones to ash dieback. *For.Pathol*.**2016**, *46*, 269–280. DOI: https://doi.org/10.1111/efp.12253
32. Cleary, M.R.; Blomquist, M.; Vetukuri, R.R.; Bohlenius, H.; Witzell, J. Susceptibility of common tree species in Sweden to *Phytophthora cactorum*, *P. cambivora* and *P. plurivora*. *For.Pathol*.**2017**, *47*, e12329. DOI: https://doi.org/10.1111/efp.12329
33. Ding, T.; Palmer, M.W.; Melcher, U. Community terminal restriction fragment length polymorphisms reveal insights into the diversity and dynamics of leaf endophytic bacteria. *BMC Microbiol*.**2013**, *13*, 1.DOI: https://doi.org/10.1186/1471-2180-13-1
34. Kovalitskaya, Y.A.; Dayanova, L.K.; Azarova, A.B.; Shestibratov, K.A. RNA interference-mediated down-regulation of 4-coumarate:coenzyme A ligase in *Populus tremula* alters lignification and plant growth. *Int. J. Envir. Sci. Educat*. **2016**, *11*, 12259–12271.

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