What Is Important in Selecting *Phlebiopsis gigantea* Strain for Commercial Use?

Zbigniew Sierota¹, Justyna Anna Nowakowska², Katarzyna Sikora¹, Marta Wrzosek³, Anna Żółciak¹, Monika Małecka¹

1. Department of Forest Protection, Forest Research Institute, Sękocin Stary, Braci Leśnej 3, Raszyn 05-090, Poland

2. Laboratory of Molecular Biology, Forest Research Institute, Sękocin Stary, Braci Leśnej 3, Raszyn 05-090, Poland

3. Faculty of Biology, University of Warsaw, Krakowskie Przedmieście 26/28, Warszawa 00-927, Poland

Abstract: *Phlebiopsis gigantea* strains registered in the European Union as biocontrol agents against root rot in forests (four from Great Britain and two from Finland) were compared using Ward's method with reference to: (1) similarity of DNA-random amplified microsatellite (RAMS) ladders, (2) cellulase and peroxidase production and (3) acceleration of dry mass wood loss in Norway spruce. The activity of the enzymes was tested in the initial phase of wood decay (30 d after inoculation) and indicated as the most active isolates: VF10 and FC15 for cellulase and FC16 and VF10 for peroxidase production. The assessment of loss of wood six months after inoculation indicated isolate FC15 as the most active. *P. gigantea* isolates similar in terms of enzyme activity indicated different patterns of DNA microsatellite loci. At the same time, DNA-RAMS revealed similarities in isolates with different abilities to produce enzymes. However, some similarities and differences between isolates according to wood decay were found. No plain relationships between molecular characteristics and enzyme activity of the strains tested were observed. The results differentiated activity of tested isolates and suggested benefits of selecting *P. gigantea* strains for commercial use basing mainly on the assessment of wood loss activity.

Key words: *Phlebiopsis gigantea* isolates, DNA-RAMS, enzyme activity, Norway spruce wood loss, Ward's Euclidean dendrograms.

1. Introduction

The biological competitor *Phlebiopsis gigantea* has been commonly used to control root rot caused by *Heterobasidion annosum* and *H. parviporum* in European coniferous stands for a long time [1, 2], but now only registered *P. gigantea* isolates can be used in forest practice within the European Union (EU) (Council Directive 91/414/EEC and Commission Regulation No 2229/2004). Consequently, no more than 14 *P. gigantea* isolates are currently used in two products (PG Suspension and commercial Rotstop) as biological control agents against root rot pathogens in conifer stands.

Mandatory EU regulations on the usage of specific *P.*

gigantea isolates have instigated research on enzyme activity of fungus mycelium used in commercial preparations. Besides, there have been carried out studies on efficacy of colonization of coniferous stumps and roots by the fungus and their resultant decomposition in Denmark [3, 4], Estonia [5], Finland [6], France [7], Germany [8], Italy [9], Latvia [10], Romania [9], Sweden [11, 12], Norway [13] and United Kingdom [14, 15]. In Poland, some of the issues concerning challenges of successful treatment have already been solved. The results obtained by Żółciak et al. [16, 17] allowed selecting *P. gigantea* strains, including registered ones, with enhanced potential to produce specific enzymes to fast decay pine and spruce wood under laboratory conditions. Activities of cellulase and peroxidase seem to be an important factor in selecting *P. gigantea* strains as roots and stumps wood decomposer in commercial preparations.

Corresponding author: Justyna Anna Nowakowska, Ph.D., assistant professor, research fields: population genetics, molecular biology, molecular identification and detection of pathogens. E-mail: J.Nowakowska@ibles.waw.pl.

Decomposition the lignin-cellulose complex in the cell wall is an important factor in wood decay efficacy. *P. gigantea* is described as “white rot” fungus since it is more concentrated on lignin decomposition of wall. Lignin and non-phenol lignin structures constitute about 90% of sub-structures of this complex polymer [18]. Kirk et al. [19] reported that lignin degradation comes into view at the end of the stage of *P. gigantea* secondary growth inside wood. Cellulose is degraded as a result of the activity of hydrolytic cellulases (glucanase and glucosidase) as well as dehydrogenases and oxidases which are produced afterwards [20, 21]. Żółciak et al. [17] found that the production of cellulase by several *P. gigantea* strains was higher when compared to other isolates tested and generally greater in the wood mycelium than that in the aerial mycelium. The authors found that average cellulase activity was the highest for Finnish isolate FV10, whereas average peroxidase activity was the highest for English isolate FC16. Cellulase production was also investigated by Niranjane et al. [22], who stressed an important role of carbon sources in activation of this enzyme. The activity of cellulase was recently tested by Li et al. [23], who compared Finnish Rotstop *P. gigantea* strain with native Chinese isolates of this fungus.

Specificity of forest location, environmental conditions as well as resultant differentiation in genetic and physiological features of coniferous stands in Poland encourage undertaking studies on *P. gigantea* strains effectiveness and their practical usefulness at a local level, in view of different climatic, habitat and management conditions [17, 24]. Since it is possible that the registered strains (active in tests performed in the country of their origin) can show the other kind of activity under different habitat conditions in another country.

The principal aim of the present study was to compare similarity of selected registered *P. gigantea* strains with regard to their molecular status, enzyme (cellulase and peroxidase) productivity and wood decay activity. The question was “what is most

important in selecting the best *P. gigantea* strain for commercial use?” To optimize the criterion of similarity, the Ward’s method with the squared Euclidean distance [25, 26] was used in hierarchical cluster analysis to sort the investigated isolates into common or separate clusters. Fungus strains were obtained from Rotstop and PG Suspension producers and represented *P. gigantea* mycelium growing on coniferous stumps in Scandinavia (Finland, Sweden) and Great Britain, respectively. It was assumed that the results of molecular analyses carried out with the use of random amplified microsatellite (RAMS) method [27-31] as well as comparisons of strain enzymatic, and wood decay activities would distinguish similarities between the strains tested and point towards the most vigorous ones, in view of treatment efficacy in practice. The results of some enzyme assays and Norway spruce wood decay assessments described below were extracted from the database described by Żółciak et al. [17].

2. Materials and Methods

2.1 *Phlebiopsis gigantea* Isolates

The six single-spore *P. gigantea* strains tested in this study originated from Finland (two marked as VF8-Rotstop, VF10) and Great Britain (four marked as FC14, FC15, FC16 (used in commercial PG Suspension) and FC17). Finish strains were isolated from *P. gigantea* fruit bodies, and those from Great Britain were isolated from *Pinus sylvestris* stumps. The description of the isolates used in the study is described by Sierota et al. [2].

2.2 DNA Extraction, Amplification, Polymerase Chain Reaction (PCR) Products and RAMS Analysis

The aerial and outer wood mycelia were removed from the wood samples. Total DNA of each isolate was extracted from 100 mg homogenized mycelium using GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). The PCR amplifications were carried out with the primer 5'-DHB(CGA)₅3' [28]

consistent with RAMS method and primers' structure as following: $D = (G/A/T)$, $H = (A/T/C)$, $B = (G/T/C)$ [27, 30]. For each DNA sample, three independent reactions were run. Subsequent to the amplification, PCR products were purified using GeneClean kit (MP Biomedicals, CA, USA), and then separated by electrophoresis in agarose Basica LE BLE100 (Prona, Spain) with SynerGel additive (diversified biotech, MA, USA) at concentrations 0.85% and 0.9%, respectively. Optimal resolution for RAMS markers of different lengths was obtained. For evaluation of band length and microsatellite occurrence matrix creation, the Genoplast Biochemicals (GBP) 3,000 bp DNA ladder (Genoplast, Poland) was used. The similarity between the isolates was assessed by comparing and counting the ladders (bp) in the matrix.

2.3 Wood Samples

Experimental wood blocks (0.5 cm × 1 cm × 2 cm) were made of hardwood collected 0.5-1.0 m above the soil level from a healthy Norway spruce tree 60-years-old growing in Ujsoły Forest District (Southern Poland, 49°28'37"N; 19°06'29"E). The weight of blocks was noted after drying at 108 °C. Later, the samples were watered to increase humidity to 70% [32]. Two and half weeks earlier, the standardized (0.25 cm²) mycelium of particular *P. gigantea* isolates was inoculated on 2% malt extract agar (MEA) in Kolle flasks and cultivated at 25 °C. The wood samples were placed in pairs (samples a and b) on the surface of growing mycelium and cultivated at 24 °C. After 30 d of incubation, samples a were removed (in 10 repetitions) with both aerial mycelium, and circa 2 mm layer of decayed wood from upper and two lateral sides of each block (hereinafter called "mycelium wood") were carefully removed from the surface and used to enzymes assay. After six months of cultivation, samples b were cleaned from aerial mycelium only, dried and weighed. The loss of dry mass of wood was counted and compared with initial dry weight [17].

2.4 Enzymes Assay

Mechanically shredded samples of mycelium wood from samples a were suspended in distilled water (1:15) and later mixed with corundum, filtered (size 6 Miracloth filter), rotated at 6,000 rpm for 10 min and assigned for enzymatic analyses. Cellulase activity (µg of reduced sugars/g dry weight/24 h) was assessed according to Pancholy and Rice [33] with the addition of carboxymethylcellulose (CMC) as the substrate. Peroxidase activity (U/g dry weight) was assessed with o-dianisidine used as the substrate [34]. Two extreme values were omitted. Methodology used in enzyme preparation and assays was presented in details by Żółciak et al. [16, 17].

2.5 Statistical Analysis

In order to discriminate the means, both the ANOVA-type III and the multiple range test and Tukey's honestly significant difference (HSD) procedures were used. Based on the matrix of bands obtained in genetic analyses as well as the mean values of tested enzyme activity and mean values of wood decay, the similarity between the isolates was tested. Ward's method with the squared Euclidean distance was used [25]. For arranging the hierarchy of clusters with high homogeneity and distinguishing similarity among *P. gigantea* isolates within clusters, when the coefficient of variation (*cv*) was greater than 15%, Staghraphics Centurion software and 2010 past free scientific software were applied [35]. The correspondence analysis was created in Statistic version 2014 software.

3. Results

3.1 Genetic Assessment

The DNA-RAMS markers indicated genetic similarity of tested isolates [2]. The present study revealed in total 20 variable RAMS markers scored from amplification profiles. Four DNA markers were observed in all isolates (+/- 1,520 bp, +/- 320 bp, +/-

270 bp and +/- 260 bp), and eight ones were unique (Table 1). Isolate FC14 was characterised by the highest number of bands (13 bands), and VF10 by the lowest (eight bands). Three of the unique bands were characteristic only for isolate FC16 (+/- 1,100 bp, +/- 880 bp and +/- 450 bp), and the other four for isolate FC14 (+/- 700 bp, +/- 300 bp, +/- 290 bp and +/- 240 bp).

By the presence of individual banding patterns in investigated *P. gigantea* isolates, the high RAMS polymorphism was indicated. The highest similarity level was found for the pair FC17-VF8, whereas the lowest similarity was shown by the pair FC14-FC16. Two isolates from Verdera, Finland (VF8 and VF10) were quite similar to each other, despite of the fact that one of them originally came from Sweden (VF10) and the second from Finland (VF8) [2] (in press).

Table 1 Occurrence of RAMS markers in tested strains; in comparison with approximate band lengths in base pairs of respective amplicons (in the first column).

Pattern (bp)	Strain code					
	VF8	VF10	FC14	FC15	FC16	FC17
1,900	1	0	0	1	0	1
1,520*	1	1	1	1	1	1
1,350	1	0	0	0	1	1
1,200	1	1	1	1	0	0
1,100	0	0	0	0	1**	0
1,000	1	0	1	0	1	1
900	1	1	0	1	1	1
880	0	0	0	0	1**	0
700	0	0	1**	0	0	0
600	0	1	0	0	0	1
490	1	1	1	1	0**	1
450	0	0	0	0	1**	0
420	0	0	1	1	0	1
400	0	0	1	1	0	0
320*	1	1	1	1	1	1
300	0	0	1**	0	0	0
290	0	0	1**	0	0	0
270*	1	1	1	1	1	1
260*	1	1	1	1	1	1
240	0	0	1**	0	0	0
Total	10	8	13	10	10	11

*Indicates pattern with the same marker represented in all strains;

**Means that only one strain differs from the rest strains.

Comparing the Ward similarity clustering, three main clusters of isolates were distinguished: (1) VF10; (2) VF8, FC15, FC16, FC17; (3) FC14 (Fig. 1).

3.2 Wood Decay

Żółciak et al. [17] showed that after six months of inoculation the lowest dry wood loss due to decay was caused by VF8 (22.5%) and the highest by FC15 (45.0%). Other isolates tested in this study showed an intermediate level of wood decay activity: 28%-39% (Fig. 2). The differences between the isolates were statistically significant.

Comparing the wood decay phenomenon, the isolates were divided into two separate clusters (Fig. 3). The first cluster comprised isolates with lower decay activity (VF8, VF10 and FC16), whereas the other one isolates with higher decay activity (FC15, FC14 and FC17).

3.3 Enzyme Activity

The VF8 strain indicated the lowest production of cellulase, whereas VF10 and FC15 showed the highest amounts of this enzyme. Even in the case of a tenfold, no statistical significance difference between mean peroxidase amounts in FC14 and FC16 mycelium was proved. Both tested enzymes produced by isolate VF10 showed a relatively high content in mycelium.

Fig. 1 Dendrogram of genetic similarity for RAMS markers of tested *P. gigantea* isolates.

Fig. 2 Dry weight wood loss (%) in Norway spruce samples after six months of inoculation with *P. gigantea* isolates.

Fig. 3 Dendrogram for comparison of wood decay caused by tested isolates of *P. gigantea*.

Table 2 Average values of cellulase and peroxidase in wood mycelium of tested *P. gigantea* isolates (ascending order).

Cellulase		Peroxidase	
Isolate	Mean*	Isolate	Mean**
VF8	9.944	FC14	0.1990
FC16	10.096	VF8	0.4165
FC14	10.399	FC15	1.1310
FC17	10.675	FC17	1.1505
FC15	12.418	VF10	1.5600
VF10	12.836	FC16	1.8745

* μg of reduced sugars/g of dry weight/24 h; **U/g of dry weight.

The results on cellulase production obtained using Ward's method (Fig. 4) showed a great similarity in the following strain pairs: VF8-FC16 and FC14-FC17 (one cluster) and VF10-FC15 (another cluster). For peroxidase production, a great enzymatic activity

resemblance was shown for the pairs: VF8-FC 14 (one cluster) and VF10-FC16, FC15-FC17 (another cluster). The comparison of the dendrograms indicated different similarity levels of the enzyme activity tested in the studied isolates.

The correspondence analysis results showed the structure of relations between *P. gigantea* isolates and their activity (Fig. 5). The quality of representation of particular data is rather high (0.896); the first eigenvalue (X) explain 55.44% of variation and the second (Y) further 34.17%. The largest contribution to the overall inertia points for isolates the isolate FC16 (harbouring the greatest distance from 0.0 X-axis) and for variants both activity of decay and amount of produced peroxidase (the greatest distance from 0.0 Y-axis). Both, wood decay and cellulase activity characterized high activity of isolate FC15. In turn, isolates VF8 and FC14 are characterised by high similarity of patterns in RAMS.

4. Discussion

Recommendation of "the best" isolate for application in any European country (Great Britain, Finland, Sweden, France, Poland) or overseas (Canada, China), as the most effective way to control root pathogens, obliges to use the most active fungus strain and the most useful formulation of the product. Now the question is what is the most "active" and "effective"

Fig. 4 Dendrograms for cellulase (a) and peroxidase (b) activity of tested isolates.

Fig. 5 Correspondence analysis for tested isolates and their activity measurement.

isolate? The present study concerns suitability of specific *P. gigantea* isolates and the results obtained allowed identification of more or less active isolates in enzymes production or/and wood decay. Also the genetic similarities and differences among some isolates were found. They suggest that the most active isolates among tested ones do not always coincide with the activity of isolates in commercial products, registered as the active substance for application in the protection against root rot pathogens in Norway spruce forests.

Under field conditions, fast stump colonization by

P. gigantea mycelium is required. This is due to variable ecophysiological factors, which may compete or impede fungal survival and thus influence treatment efficacy. In this context, most important factors are: the effects of foregoing occurrence of root pathogens in the stump (partially decomposed wood, zones of mycelium growth inhibition), excretion of resin (change of wood reaction, influence of volatile and non-volatile sesquiterpenes), fast dehydration of stump upper parts (decrease of substrate water potential needed for spore germination) [36, 37], as well as natural genetic variability of wood formation

[38].

Pratt et al. [39] described differences between commercial products containing diverse *P. gigantea* mycelia and identified differences resulting from the source of origin of maternal mycelium as well as production and application methods in European countries. The results obtained under different site conditions in Poland (i.e., climate, density of forests, age of trees, treatment timing, season of application, etc.), both in Scots pine and Norway spruce stands indicated high but differentiated efficacy of the products used in protective treatments [24]. Those results suggest that in the first years after application the effectiveness of *P. gigantea* inoculation on spruce stumps is lower than that on pine stumps.

In this study, the RAMS analysis generally revealed a high level of dissimilarity in the strains tested. Except VF8 isolate, the pattern of genetic relatedness of *P. gigantea* strains (determined based on microsatellite DNA comparisons) was not reflected in the pattern of similarities in enzymatic characteristics or else wood decay activity of the strains. The FC15 strain showed high cellulase activity, mainly due to considerable quantity of cellulase produced by its mycelium in wood as well as cellulase and dehydrogenase produced by its aerial mycelium [17]. Examination of enzyme activity of FC16 isolate used in commercial PG Suspension preparation (FOC PG B20/5, IMI 390096) indicated its moderate cellulase ability to decompose wood contrary to high peroxidase production. The other registered isolate VF8 (VRA 1835, ATCC 90304) used in commercial Rotstop also showed lower activity of wood decay and cellulase and peroxidase production. Ah Chee et al. [40] found no statistical relations between *P. radiata* wood loss and cellulase activity in liquid cultures (*P. gigantea* was included into six highest producers of this enzyme). Li et al. [23] suggested that local Chinese strains of *P. gigantea* were better to control *H. parviporum* than Rotstop. However, this remark was not confirmed by Małecka et al. [24] during studies

carried out under the environmental conditions of Polish forests.

The present study confirmed some genetic differences amongst standardized *P. gigantea* isolates, derived from different countries in Europe [41]. Vainio and Hantula [30] recently suggested even that European isolates should not be introduced into North American forests to avoid migration novel genotypes. It seems to be a discussion argument because transfer based on “the best” strains provides genetic recombination, and so to some extent, determines the adaptation of the species to environmental changes. It allows also the dispersion of strains and their ability to colonize new habitats, which may affect the positive role of this useful species to strength the native population.

The obtained results confirm the importance of an appropriate selection of *P. gigantea* strains used in commercial products based on their ability to degrade particular wood species. Moreover, preference should take into account the effect of decreasing decay activity, since fungus strains lose their properties with culture duration [42]. Multiple passage of fungus mycelium “from agar by agar to lyophilised preparation” (which is the case of *P. gigantea* commercial products manufactured by Verdera Finland and the Forestry Commission, Great Britain) can decrease its activity with time [43]. Consequently, the expiration date both of preparation and working fluid should be respected [44].

5. Conclusions

In answering the question posed: “what is important in selecting a given *Phlebiopsis gigantea* strain for commercial use”, we found that fungus strains considered as dynamic in terms of enzymes activity may differ among each other from a molecular point of view and wood decay. And *vice versa*—the isolates found to be similar at the genetic level can have completely different abilities to decay wood. Even if no regular pattern of cluster distribution between

investigated strains was observed, the FC14 isolate was defined as genetically different to the others isolates. Surprisingly, a great similarity of FC15 and FC16 to the others isolates is in opposition to the different enzymatic activity and wood decay after six months. Then again, another isolate examined (FC16; used in PG Suspension) showed moderate wood decay activity and low cellulose, but high peroxidase production. The VF8 isolate registered and used commercially in Rotstop showed the lowest cellulose and peroxidase activity as well as wood decay activity, on the contrary to a very active isolate VF10.

The results of a great similarity of isolates tested according to RAMS genetics preclude invasion fears of foreign genotypes of *P. gigantea* used as competitors of *H. annosum* in many European countries. The main factor in *P. gigantea* strains assessment regarding their biopesticidal activity, seems to be the fast and effective wood decay ability.

Acknowledgments

The study was carried out in the framework of the research project BLP-329 financed by the General Directorate of State Forests, Poland. Authors thank Dr. Marina Niemi (Verdera, Finland) and Dr. Kathy Tubby (Forestry Commission, UK) for providing pure cultures of *P. gigantea* strains used in the study.

References

- [1] Garbelotto, M., and Gonthier, P. 2013. "Biology, Epidemiology and Control of Heterobasidion Species Worldwide." *Ann. Rev. Phytopath.* 51: 39-59.
- [2] Sierota, Z., Nowakowska, J. A., Sikora, K., Wrzosek, M., Zółciak, A., and Małecka, M. 2015. "Genetic Differentiation among Wood Decay Fungus *Phlebiopsis gigantea* Strains Assessed with Random Amplified Microsatellites (RAMS) Technique." *Baltic Forestry*, Vol. 21, No. 2. (in press)
- [3] Thomsen, J. M. 2003. "Effect of Stump Treatment on Transfer of *Heterobasidion annosum* Root Rot in Norway Spruce." In *Root and Butt Rots of Forest Tree*, edited by Laflamme, G., Berube, J. A., and Bussieres, G. Ottawa: Canadian Forest Service, 170-5.
- [4] Berglund, M., and Roenneberg, J. 2004. "Effectiveness of Treatment of Norway Spruce Stumps with *Phlebiopsis gigantea* at Different Rates of Coverage for the Control of *Heterobasidion*." *For. Path.* 34 (4): 233-43.
- [5] Drenkhan, T., Hanso, S., and Hanso, M. 2008. "Effect of the Stump Treatment with *Phlebiopsis gigantea* against *Heterobasidion* Root Rot in Estonia." *Baltic Forestry* 14 (1): 16-25.
- [6] Korhonen, K., Lipponen, K., Bendz, M., Johansson, M., Ryen, I., Venn, K., Seiskari, P., and Niemi, M. 1994. "Control of *Heterobasidion annosum* by Stump Treatment with 'Rotstop'—A New Commercial Formulation of *Phlebiopsis gigantea*." In *Proceedings of VIII IUFRO Conference on Root and Butt Rot*, 675-85.
- [7] Soutrenon, A., Levy, A., Legrand, P., Lung-Escarmant, B., Guillaumin, J. J., and Delatour, C. 1998. "Comparison between Three Stump Treatments to Control *Heterobasidion annosum* (Urea, Disodium Octaborate, *Phlebiopsis gigantea*)." In *Root and Butt Rots of Forest Trees*, edited by Delatour, C., Guillaumin, J. J., Kung-Escarmant, B., and Marcais, B. France: INRA, 381-9.
- [8] Metzler, B., Thumm, H., and Scham, J. 2005. "Stubbenbehandlung vermindert das Stockfaulerisiko I Fichten-Erstaufforstungen." *AFJZ* 174: 81-4.
- [9] La Porta, N., Grillo, R., Ambrosi, P., and Korhonen, K. 2003. "Stump Treatment Experiments against *Heterobasidion annosum* in the Italian Alps." In *Root and Butt Rots of Forest Trees*, edited by Laflamme, G., Berube, J. A., and Bussieres, G. Ottawa: Canadian Forest Service, 176-80.
- [10] Treikale, O., Pugačova, J., and Afanasjeva, I. 2005. "Motivation of Fungicide Use for Intensive Oil-Seed Rape Growing Technology." In *Lauka izmēģinājumi un demonstrējumi 2005*. Ozolnieki: LLKIC, 68-74. (in Lithuanian)
- [11] Thor, M., and Stenlid, J. 2005. "*Heterobasidion annosum* Infection of *Picea abies* Following Manual or Mechanized Stump Treatment." *Scand. J. Fore. Res.* 20 (2): 154-64.
- [12] Berglund, M., Roenneberg, J., Holmer, L., and Stenlid, J. 2005. "Comparison of Five Strains of *Phlebiopsis gigantea* and Two *Trichoderma* Formulations for Treatment against Natural *Heterobasidion* Spore Infections on Norway Spruce Stumps." *Scand. J. Fore. Res.* 20 (1): 12-7.
- [13] Roenneberg, J., and Cleary, M. R. 2012. "Presence of *Heterobasidion* Infections in Norway Spruce Stumps Six Years after Treatment with *Phlebiopsis gigantea*." *Fore. Path.* 42 (2): 144-9.
- [14] Tubby, K. V., Scott, D., and Webber, J. F. 2008. "Relationship between Stump Treatment Coverage Using the Biological Control Product PG Suspension and Control of *Heterobasidion annosum* on Corsican Pine, *Pinus nigra* ssp. *Laricio*." *Fore. Path.* 38 (1): 37-46.

- [15] Webber, J. F., and Thorpe, K. V. 2003. "Potential for Biological Control of *Heterobasidion annosum* in the UK Using Rotstop®." In *Proceedings of the IUFRO International Conference on Root and Butt Rot 2001, Quebec, Canada*, 221-5.
- [16] Żółciak, A., Kornilowicz-Kowalska, T., Sierota, Z., and Iglík, H. 2008. "Enzymatic Activity of *Phlebiopsis gigantea* Isolates." *Acta Mycol.* 43 (1): 41-8.
- [17] Żółciak, A., Sierota, Z., and Małecka, M. 2012. "Characterisation of Some *Phlebiopsis gigantea* Isolates with Respect to Enzymatic Activity and Decay of Norway Spruce Wood." *Bio. Sci. Techn.* 22 (7): 777-90.
- [18] Sigoillot, C., Camarero, S., Vidal, T., Record, E., Esther, M., Pérez-Boada, M., Martínez, M. J., Sigoillot, J. C., Asther, M., Colom, J. F., and Martínez, A. T. 2005. "Comparison of Different Fungal Enzymes for Bleaching High-Quality Paper Pulps." *J. Biotech.* 115 (4): 333-43.
- [19] Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. 1978. "Influence of Culture Parameters on Lignin Metabolism by *Phanerochaete chrysosporium*." *Arch. Microb.* 117 (3): 277-85.
- [20] Henriksson, G., Ande, P., Pettersson, B., and Pettersson, G. 1995. "Cellobiose Dehydrogenase (Cellobiose Oxidase) from *Panerochaete chrysosporium* as a Wood-Degrading Enzyme: Studies on Cellulose Xylan and Synthetic Lignin." *App. Microb. Biotech.* 42 (5): 790-6.
- [21] Reddy, C. A., and D'Souza, T. M. 1998. "Application of PCR in Studying Lignocellulose Degradation by Basidiomycetes." In *Application of PCR in Mycology*, edited by Bridge, P. D., Arora, D. K., Reddy, C. A., and Elander, R. P. Wallingford, UK: CAB International, 205-42.
- [22] Niranjane, A. P., Madhou, P., and Stevenson, T. W. 2007. "The Effect of Carbohydrate Carbon Sources on the Production of Cellulase by *Phlebia gigantea*." *Enzyme and Microbial Technology* 40 (6): 1464-8.
- [23] Li, X. C., He, S. H., and Dai, Y. C. 2014. "Optimization of Submerged Culture Requirements for the Cellulase Activity by *Phlebiopsis gigantea* and Screening the Most Effective Strain." *Biotechnology Bulletin* 4: 152-8.
- [24] Małecka, M., Żółciak, A., Sikora, K., and Sierota, Z. 2012. "Evaluating the Persistence of *Phlebiopsis gigantea* (Fr.: Fr.) Julich Mycelium and Fruiting Bodies in Pine Stumps after Root-Rot Protection Treatments." *Leśne Prace Badawcze* 73 (2): 127-36. (in Polish)
- [25] Ward, J. H. 1963. "Hierarchical Grouping to Optimize an Objective Function." *J. Stat. Assoc.* 58 (301): 236-44.
- [26] Murtagh, F., and Legendre, P. 2011. "Ward's Hierarchical Clustering Method: Clustering Criterion and Agglomerative Algorithm." Accessed December 11, 2011. <http://arxiv.org/pdf/1111.6285v2.pdf>.
- [27] Ziętkiewicz, E., Rafalski, A., and Labuda, D. 1994. "Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification." *Genomics* 20 (2): 176-83.
- [28] Hantula, J., Dusabenyagasani, M., and Hamelin, R. C. 1996. "Random Amplified Microsatellites (RAMS)—A Novel Method for Characterizing Genetic Variation within Fungi." *Eur. J. For. Path.* 26 (3): 159-66.
- [29] Vainio, E. J. 1998. "Ecological Impacts of *Phlebiopsis gigantea* Biocontrol Treatment against *Heterobasidion* spp. as Revealed by Fungal Community Profiling and Population Analyses." *Dissertationes Forestales* 63, Finnish Society of Forest Science. Accessed May 2008. <http://www.metla.fi/dissertationes/df63.htm>.
- [30] Vainio, E. J., and Hantula, J. 2000. "Genetic Differentiation Between European and North American Populations of *Phlebiopsis gigantea*." *Mycologia* 92 (3): 436-46.
- [31] Zakaria, L., Kulaveraasingham, H., Tan, S. G., Abdullah, F., and Ho, Y. W. 2005. "Random Amplified Polymorphic DNA (RAPD) and Random Amplified Microsatellite (RAMS) of *Ganoderma* from Infected Oil Palm and Coconut Stumps in Malaysia." *Asia Pacific J. Mol. Biol. and Biotech.* (13) 1: 23-34.
- [32] Sierota, Z. 1997. "Dry Weight Loss of Wood after the Inoculation of Scots Pine Stumps with *Phlebiopsis gigantea*." *Eur. J. For. Path.* 27 (3): 179-85.
- [33] Pancholy, S. K., and Rice, E. L. 1973. "Soil Enzymes in Relation to Old Field Succession: Amylase, Cellulose, Invertase, Dehydrogenase and Urease." *Soil Sci. Soc. Am. J.* 37 (1): 47-50.
- [34] Maehly, A. C., and Chance, B. 1954. "The Assay of Catalases and Peroxidases." In *Methods of Biochemical Analysis*. Vol. 1, New York: Interscience, 358-423.
- [35] Hammer, Ø., Harper, D. A. T., and Ryan, P. D. 2001. "Past: Paleontological Statistics Software Package for Education and Data Analysis." *Palaeontologia Electronica* 4 (1): 9. Accessed May 21, 2001. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.
- [36] Redfern, D. B. 1993. "The Effect of Wood Moisture on Infection of Sitka Spruce Stumps by Basidiospores of *Heterobasidion annosum*." *Eur. J. For. Path.* 23 (4): 218-35.
- [37] Sierota, Z. 1995. "The Role of the Fungus *P. gigantea* as a Limiting Factor of *H. annosum* in the Scots Pine Stands in Post-Agricultural Lands." *Forest Research Papers* 10, 180. (in Polish)
- [38] Wodzicki, T. J. 2001. "Natural Factors Affecting Wood Structure." *Wood Sci. Tech.* 35 (1-2): 5-26.
- [39] Pratt, J., Niemi, M., and Sierota, Z. 2000. "Comparison of Three Products Based on *Phlebiopsis gigantea* for the

- Control of *Heterobasidion annosum* in Europe.” *Bio. Sci. Techn.* 10 (4): 467-77.
- [40] Ah Chee, A., Farrell, R. L., Stewart, A., and Hill, R. A. 1998. “Decay Potential of Basidiomycete Fungi from *Pinus radiata*.” In *Proceedings of 51st New Zealand Plant Protection Conference*, 235-40.
- [41] Kauserud, H., and Schumacher, T. 2003. “Regional and Local Population Structure of the Pioneer Wood-Decay Fungus *Trichaptum abietinum*.” *Mycologia* 95 (3): 416-25.
- [42] Pandey, K. K., and Pitman, A. J. 2003. “FTIR Studies of the Changes in Wood Chemistry Following Decay by Brown-Rot and White-Rot Fungi.” *Intern. Biodeter. Biodegr.* 52 (3): 151-60.
- [43] D’Souza, T. M., Merritt, C. S., and Reddy, S. A. 1999. “Lignin-modifying Enzymes of the White Rot Basidiomycete *Ganoderma lucidum*.” *Appl. Environ. Microbiol.* 65 (12): 5307-13.
- [44] Thor, M., Bendz-Hellgren, M., and Stenlid, J. 1997. “Sensitivity of Root Rot Antagonist *Phlebiopsis gigantea* Spores to High Temperature or Pressure.” *Scand. J. For. Res.* 12 (4): 356-61.