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Interactions between embryogenic callus of *Abies alba* and *Heterobasidion* spp. in dual cultures

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Abstract

Interactions between three genotypes of a silver fir (*Abies alba* Mill.) embryogenic callus and *Heterobasidion abietinum*, *H. parviporum*, and *H. annosum* were examined in dual cultures. The aim of this study was to determine whether dual cultures can be used to evaluate the degree of fungal virulence at an embryogenic level, and whether different genotypes of a callus show different susceptibility. The dual cultures were performed on Schenk and Hildebrandt medium. Mycelial growth of *H. parviporum* and *H. annosum* was significantly stimulated in the presence of the callus but was not directional in nature. The embryogenic callus died between six and nine days after being colonized by *H. parviporum* suggesting that this was the most virulent species. By contrast, the callus remained healthy for up to 50 d after colonization with *H. abietinum* suggesting that this was the least virulent species. The callus of the *A. alba* genotype which originated in the mountain region of Poland remained healthy and alive for a significantly longer period than the other two genotypes in the dual cultures with all three *Heterobasidion* species even though overgrown by mycelium suggesting that the mountain genotype had the strongest defence response to *Heterobasidion* infection.

Additional key words: *in vitro* culture, mycelial growth, silver fir

Introduction

Species of the genus *Heterobasidion* are important pathogens causing root and butt rot of forest trees in Europe, North America, and Asia. They pose a threat to the sustainability of forest ecosystems, concurrently affecting the economic aspect of the management (Bendz-Hellgren and Stenlid 1997, Woodward *et al.* 1998). *Heterobasidion annosum sensu lato* is a species complex consisting of several closely related species of which three species occur in Europe: *Heterobasidion abietinum* Niemelä and Korhonen, *Heterobasidion annosum sensu stricto* and *Heterobasidion parviporum* Niemelä and Korhonen (Niemelä and Korhonen 1998). Although these three species have a partly overlapping geographic distribution, they differ in host preferences and show variations in their degree of virulence (Korhonen 1978, Capretti *et al.* 1990, Werner and Łakomy 2002, Łakomy and Werner 2003). In southern and central Europe, *H. abietinum* is specialized towards *Abies* species (Capretti *et al.* 1990, Kowalski and Łakomy 1998, Łakomy *et al.* 2007). *H. annosum sensu stricto* shows a preference for *Pinus* species, but attacks tree species belonging to *Picea*, *Alnus*, *Betula*, and *Juniperus* genera. *H. parviporum* mainly attacks trees in the genus *Picea*, but in some cases also young *Pinus sylvestris* growing around infected spruce stumps. *Abies* species, including the silver fir (*Abies alba* Mill.), are mostly infected by *H. abietinum*, whereas an infection by *H. annosum sensu stricto* or *H. parviporum* is rare. In southern Europe (Italy, Spain, and Greece) (Tsopelas and Korhonen 1996, La Porta *et al.* 1997, Sánchez *et al.* 2007) and Eurasia (Turkey) (Otrosina *et al.* 1993), where firs often suffer summer drought, *H. abietinum* can cause serious root and butt rot disease in *A. alba* (Ostrosina *et al.* 1993), *Abies pinsapo* Boiss. (Sánchez *et al.* 2007), *Abies cephalonica* Loudon, *Abies borisii-regis* Mattf. (Tsopelas and Korhonen 1996), and *Abies nordmanniana* (Steven) Spach (Doğmuş-Lehtijärvi *et al.* 2007). In contrast in central Europe, *H. abietinum* does not cause major problems in silver fir (Korhonen *et al.* 1998, Korhonen and Holdenrieder 2005), probably because the climatic conditions are less optimum for the fungus and hence the fungus is less prevalent which is reflected in the better condition of the firs in this area (Korhonen and Holdenrieder 2005).

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Abbreviations: BA - benzylaminopurine; 2,4-D - 2,4-dichlorophenoxy acetic acid; KIN - kinetin.
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The development of tissue culture techniques for various tree species has provided many possibilities for their micropropagation. An embryogenic or non-embryogenic callus may also be used for the preparation of secondary metabolites, analyses of somaclonal variation (Nawrot-Chorabik 2009), genetic transformations, and in plant pathology research. Studies on interactions between pathogens and host plant tissues allow to understand mechanisms of plant defence to infections. This knowledge is the basis of new strategies for selecting pathogen-resistant plants (Walters *et al*. 2007). Dual cultures of tree callus and fungi were first used to study host-pathogen interactions by Hřib and Rypáček (1978). They showed that the growth reactions of plant tissue cultures to the presence of fungi could be used to demonstrate the degree of their aggressiveness. Such set of defence reactions in plants have been formed in the early stages of embryonic development (Hřib *et al.* 1995).

Subsequent studies of fungus-plant tissue interactions in dual cultures investigated the interactions between fungi and beech (Hendry *et al.* 1993), spruce (Sirrenberg *et al.* 1995, Kvaalen and Solheim 2000, Kvaalen *et al.* 2001, Nawrot-Chorabik *et al.* 2011), fir (Vookova *et al*. 2006), and pine calli (Niemi *et al.* 1998). Hřib and Rypáček (1981) and Kvaalen and Solheim (2000) investigated whether fungal virulence identified at the embryogenic stage in a dual culture was correlated with the susceptibility of trees to infection. Based on their experiments, Kvaalen and Solheim (2000) observed that the susceptibility of spruce clones to fungal pathogens *in vitro* correlate with their response to infection *in vivo*.

Studies of pathogen-host tissue interactions is a part of the strategy for selecting pathogen-resistant plants (Garcion *et al.* 2007). However, *in vitro* studies conducted in dual cultures require some refinement. There are few reports concerning the influence of dual cultures on the growth response of some of the fungi with different ecological status in relation to plant cells (*i.e*., at the embryonic level). It is known that in dual fungal cultures with antagonistic fungi, the *Heterobasidion* genus produces cyclopentabenzopyranone which is a polycyclic organic compound (Sonnenbichler *et al.* 1983). The aim of this study was to increase our knowledge of the host-pathogen interaction in dual cultures with various genotypes of fir. The purpose of this study was to demonstrate the differences between the examined genotypes and to show the advantages of using tissue cultures in testing multiple fungal strains against genetically identical host material which is difficult to conduct otherwise.

Materials and methods

Zygotic embryos of silver fir were used as the primary explants for *in vitro* callus culture using somatic embryogenesis. Each set was represented by 100 explants from which three lines of embryogenic callus with the highest proliferation coefficient (the ratio between mass gain and the diameter of the callus at intervals between passages onto fresh media) representing three separate genotypes were selected for further examinations (Nawrot-Chorabik 2008, 2012). Genotype G4 originated from Nowy Targ, G5 originated from Skarżysko-Kamienna, and G6 originated from Janów Lubelski.

The tests were performed using one isolate for each of three European *Heterobasidion* species which were isolated from the roots of a dead *A. alba* tree in the Ojców National Park in Poland (50º 12' N, 19º 46' E). The isolates were identified using molecular methods (Kraj and Kowalski 2010) as: *Heterobasidion abietinum* isolate No. HMIPC 17076, *Heterobasidion parviporum* isolate No. HMIPC 18307/1, and *Heterobasidion annosum* isolate No. HMIPC 17086. The isolates were cultured for 21 d on 2 % (m/v) malt-extract agar (MEA). Subsequently, each of the isolates was subcultured onto a Schenk and Hildebrandt (1972; SH) medium further used in the dual culture studies.

Mature silver fir seeds were disinfected in 10 % (m/v) sodium hypochlorite (15 min) and imbibed in 5.0 % (m/v) polyvinylpyrrolidone (PVP) at 4 ºC for 24 h (Nawrot-Chorabik 2012). Mature zygotic embryos were excised from the seeds and placed in Petri dishes, five embryos in each, on an SH medium supplemented with 2.5 mg dm^{-3} benzylaminopurine (BA), 1 mg dm^{-3} kinetin (KIN), 0.04 mg dm^{-3} 2,4-dichlorophenoxy acetic acid (2,4-D), 5.0 mg dm^{-3} thiamine, 0.5 mg dm^{-3} pyridoxine, 5.0 mg dm^{-3} nicotinic acid, 20.0 g dm^{-3} sucrose, 1.5 g dm^{-3} casein hydrolysate, and 4.0 g dm^{-3} *Phytagel*. The callus culture was incubated in darkness at a temperature of 25 ± 1 ºC and a 50 % relative humidity. After the initiation of the embryogenic cultures, the callus was subcultured every two weeks to a fresh SH medium supplemented with an increased concentration of growth regulators (5.0 mg dm^{-3} BA, 2.0 mg dm^{-3} KIN, and 0.08 mg dm^{-3} 2,4-D) to encourage callus proliferation. The callus was stained with acetocarmine. The degree of its embryogenesis was verified by interference microscopy (nuclei in the cells of the proembryo in the proembryo-genic mass zones were stained red by acetocarmine) (Gupta and Durzan 1987).

Inocula (5.0 × 5.0 mm) were excised from 10-d-old fungal cultures growing on an SH medium and placed in the centre of a 10-cm diameter Petri dish on an SH medium supplemented with 5.0 mg dm^{-3} BA, 2.0 mg dm^{-3} KIN, and 0.08 mg dm^{-3} 2,4-D. A silver fir embryogenic callus (approximately 500 mg and about 10 mm in diameter) was placed 0.5 cm from the edge of the dish at a distance of 25 mm from the edge of the fungal inoculum. The dual cultures were incubated in the dark at 25 ± 1 ºC and a relative humidity of 50 %. Each of the three *Heterobasidion* species was paired with each line

(genotype) of the embryogenic callus, with five replicates per treatment. Controls comprised *Heterobasidion* species grown on a medium without any callus, and each callus genotype (G4, G5, and G6) grown on a medium without a fungal isolate. Mycelial growth from the inoculum towards the callus and in the opposite direction towards the edge of a dish was measured every 24 h. The growth rate of the fungi on the control plates was also measured. The morphology of the callus was observed macro- and micro-scopically every 24 h and any phenotypic changes noted (Table 1). A mycelial growth rate and phenotypic changes were used as indicators of a reaction to the presence of the callus in the dual culture.

To determine whether the mycelial isolates were inhibited or stimulated by the callus, the growth rate of the mycelia in the dual culture (towards the callus and away from it) was compared to the mycelial growth rate on the control plates using the Student's *t*-test for dependent variables. An *ANOVA* analysis using the Kruskal-Wallis test was performed to compare the fungal isolates in terms of average mycelial growth towards the callus and away from it over the course of the experiment. To identify whether the callus genotypes differed significantly between each other in terms of how long they remained alive after being colonized by the fungus, or in terms of the average time of fungal colonization, the Tukey multiple comparisons test was performed, preceded by the one-way Friedman *ANOVA* test. Calculations were performed separately for each of the fungi. All statistical analyses were conducted using *STATISTICA v. 7.1.* (*StatSoft*, Tulsa, OK, USA).

Results

The *in vitro* culture produced a high frequency of silver fir embryogenic callus initiation (15.0 %) on the SH medium (Fig. 1). The calluses in the dual cultures with *H. parviporum* and *H. annosum* were overgrown by mycelia after 7 and 9 d, respectively. However, it took on average 25 d for the *H. abietinum* mycelium to colonize the living callus. When the mycelia initially overgrew the living callus, no phenotypic changes in callus morphology were observed. Where the callus in the dual cultures with *H. parviporum* was partially covered with the mycelium, the cells were dehydrated and strongly deformed, plasmolysed, collapsed, and even necrotic. By contrast, in the dual cultures with *H. annosum*, the cells were less dehydrated, not deformed, and remained alive longer even though the mycelium of *H. annosum* covered the living callus tissue more abundantly (Table 1). The callus growing in the dual culture with *H. abietinum* remained alive and in apparently good physiological condition longer than the callus in the dual cultures with the other *Heterobasidion* species: the callus died after

Table 1. The susceptibility of embryogenic *Abies alba* callus genotypes to *Heterobasidion* species when grown in the dual culture. S - start of mycelial colonization, CD - callus death, LC - a living callus colonized by mycelium.

Fungal species	Genotype	Time since inoculation [d]					Phenotypic changes in mycelium and callus morphology
		S	CD		LC		
Heterobasidion abietinum	G4	6	56	33	50	25	The callus remained healthy for 50 d (well hydrated and white). Callus cell structure is loose. The callus rapidly overgrown by mycelium after about 10 d.
	G5	7	27	33	14	25	The mycelium overgrew the whole plate by day except for the callus surface. The callus surface became slightly overgrown on day 14.
	G6	5	16	33	11	25	The callus developed a brown coloration demonstrating symptoms of necrosis. The mycelium was densest next to the callus and the callus was completely overgrown.
Heterobasidion parviporum	G4	4	13	12	9	7	The callus became partially dehydrated, plasmolysed, developed a darkened coloration, and was partially overgrown with the mycelium. The mycelium developed a yellow coloration around the edge of the plate, except in the region of the callus.
	G5	5	11	12	6	7	The callus became severely dehydrated and showed signs of necrosis. The surface of callus was slightly overgrown with the mycelium. The mycelium developed a yellow coloration around the edge of the plate, except for in the region of the callus.
	G6	4	12	12	6	7	The mycelium grew rapidly towards the callus and was densest around the callus. The callus darkened and died.
Heterobasidion annosum	G4	6	23	16	17	9	The genotype callus G4, G5, G6 dehydrated gradually. The callus was overgrown with the mycelium. The mycelium was densest around the callus.
	G5	7	12	16	4	9	
	G6	7	13	16	6	9	

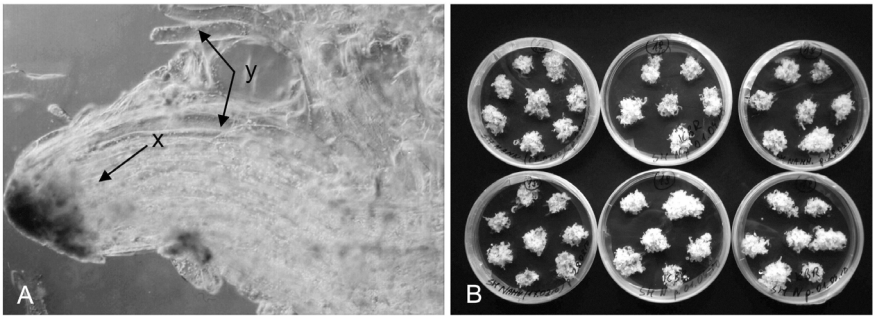

Fig. 1. *A* - A somatic pro-embryo in an *Abies alba* embryogenic callus (G4 genotype; x - a proebryogenic region, v - suspensor cells). *B* - Proliferated clones of an embryogenic callus used to establish a dual culture.

Table 2. *Heterobasidion* mycelial growth in the presence and absence of an *Abies alba* callus. The comparison of growth towards the callus from the point of inoculation, and of growth in the opposite direction towards the edge of the plate, and of inoculated and control calli. * - Differences statistically significant at $\alpha = 0.05$.

Fungal species	Direction of mycelial growth	Mean growth [mm]	Comparison of growth in different directions	Difference	α	Average time of colonization of living callus [d]			*ANOVA*	
						G4	G5	G6	*F*	*P*
Heterobasidion abietinum	control	20.28	towards – away from callus	0.434		50[a]	14[b]	11[b]	385.364	0.0000*
	towards callus	20.76	towards callus – control	0.480						
	away from callus	21.19	away from callus – control	0.914						
Heterobasidion parviporum	control	19.52	towards callus – away from callus	0.070		9[a]	6[b]	6[b]	13.500	0.0060*
	towards callus	24.01	towards callus – control	4.490	*					
	away from callus	23.94	away from callus – control	4.420	*					
Heterobasidion annosum	control	20.96	towards callus – away from callus	0.029		17[a]	4[b]	6[b]	147.000	0.0000*
	towards callus	22.82	towards callus – control	1.862	*					
	away from callus	22.79	away from callus – control	1.939	*					

33 d, corresponding to the time when the majority of the embryogenic cells die in the absence of sub-culture to a fresh medium. The callus growing in the dual culture with *H. parviporum* or with *H. annosum* died after 12 and 16 d, respectively. The calli of all the control genotypes (G4, G5, and G6) were alive and remained embryogenic until the end of the experiment (Table 1).

The embryogenic callus originating from Nowy Targ Forest District (callus line G4) remained in a good condition, well hydrated, with cells in the proembryogenic mass zones visible under the microscope for 50 d (Fig. 1). These masses constituted small callus cells, whose nuclei were stained red by acetocarmine, and long colourless suspensor cells, with small nuclei and large vacuoles. The colonization of G4 callus by the three *Heterobasidion* spp. began on days 4 to 6, similarly as observed with genotypes G5 and G6 which were colonized by the *Heterobasidion* spp. on days 5 to 7 (Table 1).

The growth rates of *H. abietinum* in the dual cultures (both towards the callus and away from it) and on the control plates were not significantly different (Fig. 2, Table 2). The *H. parviporum* mycelium in the dual cultures exhibited significantly more intensive growth ($P < 0.05$) compared with the mycelium on the control plate. The *H. annosum* mycelium also showed significantly better growth ($P < 0.05$) in the presence of the callus but to a lesser extent than the *H. parviporum* mycelium (Table 2).

ANOVA and the Kruskal-Wallis test show that the direction of *H. abietinum* mycelial growth from the point of inoculation towards the callus and in the opposite direction towards the edge of the Petri dish was not significantly different. There was also no significant difference between mycelial growth in the dual culture and on the control plates. Mycelial growths of *H. parviporum* and *H. annosum* were significantly greater

in the presence of a callus compared to the control plates, however, in the presence of a callus, the direction of growth towards or away from the callus was not significantly different (Table 2). The combined results of the non-parametric, one-way Friedman *ANOVA* test (summarized in Table 2) show that the G4 callus in the dual culture with either *H. abietinum*, *H. parviporum*, or *H. annosum* remained alive for a significantly longer period after the colonization by the mycelium than the calli of the G5 and G6 genotypes (Table 2).

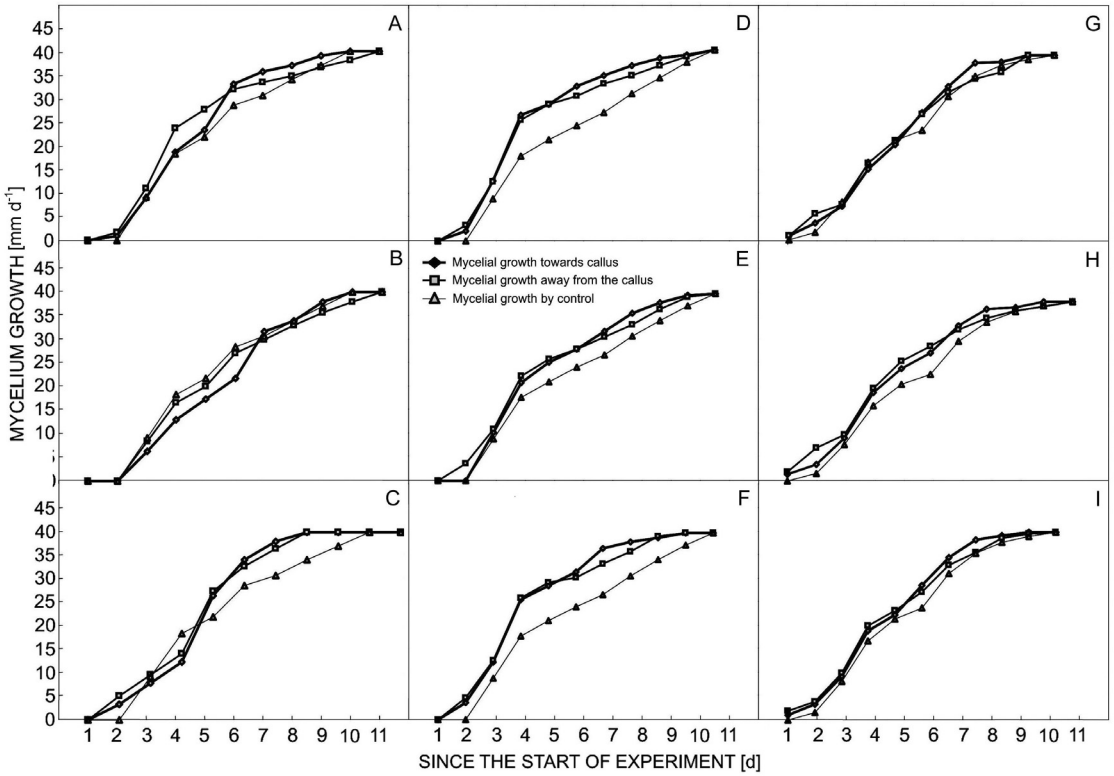

Fig. 2. The comparison of mean growth rates of *Heterobasidion abietinum* (*A*, *B*, *C*), *H. parviporum* (*D*, *E*, *F*), and *H. annosum* (*G*, *H*, *I*) mycelia in the presence of an embryogenic callus of *Abies alba* genotypes G4 (*A*, *D, G*), G5 (*B*, *E*, *H*), and G6 (*C*, *F*, *I*) compared with the growth rate of the control mycelium.

Discussion

Genetically identical tissue culture material allowed to evaluate three fir genotypes with respect to three *Heterobasidion* strains derived from naturally infected trees in order to select the potential host and pathogen clones that could be used for complex transcriptome and proteome analyses. The conducted dual-culture analyses enabled to demonstrate that the tissue cultures of the different fir genotypes actually showed differences in response to the applied fungal strains, and to select initial (or any) differences between the *Heterobasidion* species at the embryonic level. The study also showed that these differences are correlated with *ex vitro* virulence of these fungi. The greatest tolerance of the callus tissue was detected to the presence of *H. abietinum*, as the fir callus of all the examined genotypes remained healthy for up to 50 d (Table 1). This is particularly evident in the case of the embryogenic callus initiated from the G4 line. A lower resistance of the fir callus was demonstrated to the *H. annosum* mycelium, and the lowest to the *H. parviporum* (Table 2, Fig. 2). The high resistance of the embryogenic callus originating from a mountain area forest district (G4) might reflect its adaptation to the harsh conditions in its native habitat (Western Beskid, 1000 m above sea level, an average annual temperature of 7 ºC). This resistance could also result from other inherited traits that are characteristic of this genotype. As the callus of the G4 genotype showed the strongest defence against the mycelium of the three fungal species, both arguments may be true or even may be harmonized, as result of evolutionary adaptations of fir.

Another aspect to consider is the fungal pathogenicity against the host plants. The stimulation of mycelium growth of *H. parviporum* in the presence of a callus, or the lack of a callus effect on the mycelium growth of *H. abietinum* corresponded well with their pathogenicity under natural conditions. *H. parviporum* causes large losses in spruce tree stands all over the world, whereas *H. abietinum,* which occurs in central Europe, the

Pyrenees, and north-eastern Turkey, is considered to be a saprotroph or a weak parasite living mainly on stumps and dead fir logs (Munda 1994, Łakomy *et al*. 2000, Dogmus-Lehtijarvi *et al*. 2007). When *Laetiporus sulphureus* and *H. parviporum* are grown in dual cultures on nutrient-poor media, *L. sulphureus* is parasitized by *H. parviporum* (Hettich *et al*. 2007). This demonstrates the high competitive ability of *H. parviporum*.

It should be noted that callus cultures are composed of non-differentiated cells, whereas defence reactions of conifers are multi-tiered and involve both constitutive (*e.g*., lignosuberized bark, lignin-coated secondary cell walls in tracheids) and induced mechanisms (*e.g*., *de novo* synthesized phenols and terpenes). Bearing this in mind, the current study shows that *H. parviporum* could be more virulent to silver fir than *H. abietinum* despite the fact that *A. alba* is the major host for the latter fungal species. This was also confirmed by the statistical analysis. The measurements of the changes in the mycelial growth rate were also an important aspect of this study (Fig. 2). Due to the fact that the change in the growth rate could have resulted from the chemical defence of tissues (Kvaalen and Solheim 2000), it was necessary to adjust the method of analysis to this variability. Statistically significant differences were shown in the case of cultures with *H. parviporum* and *H. annosum* mycelia, strengthening the idea that *H. parviporum* should be treated as pathogen to embryogenic callus of silver fir, whereas *H. abietinum* may possibly play a role of a saprotroph. This conclusion can therefore be considered a new discovery in the perspective of pathogenicity research at the embryonic level. Therefore, when continuing this study in the future, it would be interesting to decipher the molecular basis of differences between host plants by using multiple *Heterobasidion* strains. Transcript profiling hallmark defence genes (*e.g*., Karlsson *et al*. 2007) and biochemical analyses of immune proteins, which function as transcription factors with DNA, should be useful in this study (Nawrot-Chorabik 2013). Due to the fact that different strains of one fungal species may vary in virulence, further proceedings in dual cultures involving *Heterobasidion* fungi may consolidate and expand our knowledge on this subject and make a valuable contribution to this field of research.

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