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# THE EFFECT OF A MIXTURE OF *PHLEBIOPSIS GIGANTEA* (FR.) JÜLICH METABOLITES ON MYCELIUM GROWTH OF *HETEROBASIDION* SPP. *IN VITRO*

Kamila Płońska-Kaczor[1], Piotr Łakomy[2]✉

[1]Department of Forest Utilization, Poznań University of Life Sciences
Wojska Polskiego 71A, 60-625 Poznań, **Poland**
[2]Department of Forest Pathology, Poznań University of Life Sciences
Wojska Polskiego 71C, 60-625 Poznań, **Poland**

## ABSTRACT

**Introduction.** Biological control against *Heterobasidion* root rot utilises the natural antagonistic properties of a saprotrophic fungus *Phlebiopsis gigantea* (Fr.) Jülich.
**The aim of the study** was to determine the effect of *P. gigantea* metabolites on mycelium growth in three *Heterobasidion* species found in Poland.
**Methodology.** Analyses were conducted on nine isolates of *Heterobasidion* and three isolates of *P. gigantea.*
**Results.** Secondary metabolites of the saprotroph exhibits the strongest effect on the reduction of mycelium growth in *H. annosum* and *H. parviporum,* while this effect was weakest on the mycelium of *H. abietinum*. Analysis of variance showed significant differences between mean mycelium size in individual experimental combinations ($P < 0.00001$), while based on Tukey's test 8 homogenous groups may be distinguished in terms of the effect of metabolites of *P. gigantea* isolates on individual *Heterobasidion* isolates.
**Conclusions.** It was found that secondary metabolites of *P. gigantea* isolates vary in their effect on the reduction of mycelium growth of *Heterobasidion* spp. *in vitro*.

**Keywords:** *Phlebiopsis gigantea*, *Heterobasidion* spp., secondary metabolites, biological control

## INTRODUCTION

*Heterobasidion* root rot, also referred to as annosus root rot, is one of the most economically important diseases of forest trees. It is caused by fungi from the genus *Heterobasidion*, represented in Poland by three species: *H. annosum* (Fr.) Bref, *H. parviporum* Niemelä and Korhonen, and *H. abietinum* Niemelä and Korhonen. Economic losses resulting from the presence of pathogens are connected mainly with a reduction in tree stocking and current growth increment, a deterioration of stand abundance as well as timber losses, particularly in spruce and fir stands subject to root rot (Łakomy and Werner, 2003; Woodward et al., 1998). Another important aspect is related with the need of species conversion in the stands, which have lost their stability as a result of pathogen activity. Sierota and Zachara (2011) estimated that in Poland financial losses caused by root rot amount to millions of PLN.

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✉plakomy@up.poznan.pl, https://orcid.org/0000-0002-1510-6217

Conditions promoting disease development are found in coniferous stands of first and second generations growing on former arable soil, as well as monocultures, particularly pine and spruce. *Heterobasidion* spp. attack also other coniferous species (e.g. fir, larch, Weymouth pine, etc.), while less frequently deciduous species (Sierota, 1987; 1997a; Woodward et al., 1998).

Prevention plays a significant role in the control system against *Heterobasidion* root rot. It is crucial to maintain conditions promoting root system development, thus at planting roots may not be overdry or damaged. It is recommended to perform soil cultivation operations, which would not disturb infested roots from the previous stand generations, potentially constituting a source of secondary infection. It is also necessary to maintain species diversity in afforestation and regeneration to prevent new trees' generation more susceptible to infestation (Sierota and Szczepkowski, 2014).

The above-mentioned prevention methods to control *Heterobasidion* root rot have been supplemented with biological method. A fungus *Phlebiopsis gigantea* (Fr.) Jülich, exhibiting natural antagonistic properties, is a species used for this purpose. Commercial biopreparations based on mycelia or spores of this saprotroph are used in many countries (Łakomy, 2001; Pratt et al., 2000; Sierota, 1995; 1997b; 2001; Rishbeth, 1961; 1963).

Control against *Heterobasidion* root rot focus on stands growing on former arable soil, in which stumps are protected using *P. gigantea* mycelia in various treatment variants ranging from all the stumps on the site (Sierota, 1995) to scattered spot treatment (Sierota and Małecka, 2004a) or artificial gaps (Sierota and Małecka, 2004b), depending on the situation.

Apart from the direct effect of competition for the ecological niche by fungi, the indirect action of fungal metabolites or their derivatives is also proposed as a measure in the biological protection against disease (Łakomy, 2004; Szwajkowska-Michałek et al., 2012; Kwaśna et al., 2013). Łakomy (2004) showed that species of saprotrophic fungi degrading broadleaved wood produced metabolites, which inhibited the development of mycelia and rhizomorphs of *Armillaria* species *in vitro.* The strongest effect was observed for *Bjerkandera adusta* isolates, which almost completely inhibited the development of certain *Armillaria* species. However, no such effect was detected during in vivo experiments. In turn, Szwajkowska-Michałek et al. (2012) and Kwaśna et al. (2013) suggested that the use of *Penicillium adametzi* metabolites may be used to reduce the development of both *Heterobasidion* and *Armillaria.*

When applying individual fungal isolates as agents in biological plant protection their specific properties need to be taken into consideration. In the case of *P. gigantea* it is indicated that isolates may significantly differ in terms of their wood degradation capacity (Łakomy and Zarakowski, 2000; Sierota et al., 2015) and enzymatic activity of cellulase, peroxidase, phosphatase and dehydrogenase (Sierota et al., 2015; Żółciak et al., 2008; 2012). For this reason the fungal isolates used in biopreparations need to be continuously evaluated in terms of their efficacy.

The aim of the study was to determine the effect of *P. gigantea* metabolites on mycelium growth in individual *Heterobasidion* species under laboratory conditions, assuming that these fungi interact in the natural environment colonising simultaneously the same substrate, or under the purposeful introduction of *P. gigantea* in the form of a biopreparation.

## MATERIALS AND METHODS

Analyses were conducted on nine fungal isolates of three *Heterobasidion* species (Table 1) and three isolates of *Phlebiopsis gigantea* (Table 2).

In order to obtain a mixture of metabolites from each isolate of *P. gigantea* the mycelium was cultured on liquid potato dextrose agar medium (PDA, Merck) in 5 replications. After 30 days the metabolite mixture was filtered through blotting paper and poured to Erlenmeyer flasks (with 400 ml per flask). In order to solidify the filtrate it was supplemented with agar (8 g/400 ml medium) and next sterilised (1 atm, 120°C, 20 min) and poured to Petri dishes (Łakomy, 2004). After medium solidification it was inoculated with *Heterobasidion* isolates. Inoculation was performed under sterile conditions (in an AURA 2000 laminar air flow cabinet). Each experimental combination (medium with *P. gigantea* + *Heterobasidion* sp. metabolites) was designed in five replications.

Culture diameter measurements (two cross-wise measurements) were taken after 3, 5, 7 and 10 days.

**Table 1.** Origin of *Heterobasidion* spp. isolates
**Tabela 1.** Pochodzenie izolatów *Heterobasidion* spp.

| Kod izolatu<br>Isolate code | Gatunek – Species | Gospodarz – Host | Lokalizacja – Location |
|---|---|---|---|
| 3/1/1/3 | *H. annosum* | *P. sylvestris* (stump) | Skwierzyna<br>(52°32'N, 15°21'E) |
| 03/7 | *H. annosum* | *P. sylvestris* (stump) | Człopa<br>(53°09'N, 16°07'E) |
| 5/2 | *H. annosum* | *F. sylvatica* (dead tree) | Łobez<br>(53°40'N, 15°38'E) |
| Suwałki I | *H. parviporum* | *P. abies* (stump) | Suwałki<br>54°16'N, 22°51'E |
| Suwałki II | *H. parviporum* | *P. abies* (stump) | Suwałki<br>54°16'N, 22°51'E |
| 17004 | *H. parviporum* | *P. abies* (living tree) | Świerklaniec<br>(50°25'N, 19°01'E) |
| 20056 | *H. abietinum* | *P. abies* (stump) | Nowy Targ<br>(49°25'N, 20°05'E) |
| 96070 | *H. abietinum* | *A. alba* (stump) | Węgierska Górka<br>(49°42'N, 19°15'E) |
| 17021A | *H. abietinum* | *A. alba* (lying log) | Ojcowski Park Narodowy<br>(50°12'N, 19°40'E) |

**Table 2.** Origin of *Phlebiopsis gigantea* isolates
**Tabela 2.** Pochodzenie izolatów *Phlebiopsis gigantea*

| Numer<br>Number | Gatunek – Species | Gospodarz – Host | Lokalizacja – Location |
|---|---|---|---|
| Pg | *P. gigantea* | *P. sylvestris* (stump) | Zielonka<br>(52°30'N, 17°02'E) |
| PgS | *P. gigantea* | *P. abies* (stump) | Suwałki<br>(53°59'N, 23°02'E) |
| Pg 133/10/3 | *P. gigantea* | *P. sylvestris* (stump) | Człopa<br>(53°05'N, 16°13'E) |

The control comprised mycelia cultured on the medium containing no metabolites (in 5 replications). The extent of the action of *P. gigantea* metabolites on mycelium growth in *Heterobasidion s*pp. was determined based on the percentage reduction of its size in relation to the control cultures.

Differences between mycelium growth in individual isolates of *H. annosum* in the controls and under the influence of *P. gigantea* metabolites were established using the one-way analysis of variance (ANOVA) and Tukey's HSD tests. All the analyses were performed using the Statistica 8.0 software (Dell, Round Rock, TX, USA).

## RESULTS

Metabolites of *P. gigantea* to the greatest extent inhibited mycelium growth in *H. annosum*, while to the lowest extent – in *H. abietinum* (Fig. 1). Mycelium of *H. annosum* after 7 days of growth was by 54% smaller than that of the control, while cultures of *H. parviporum* and *H. abietinum* were by 40% and 28% smaller. The diameter of *H. annosum* cultures growing in the presence of *P. gigantea* metabolites was by 38% up to 67% smaller (mean 54%) compared to the control cultures.

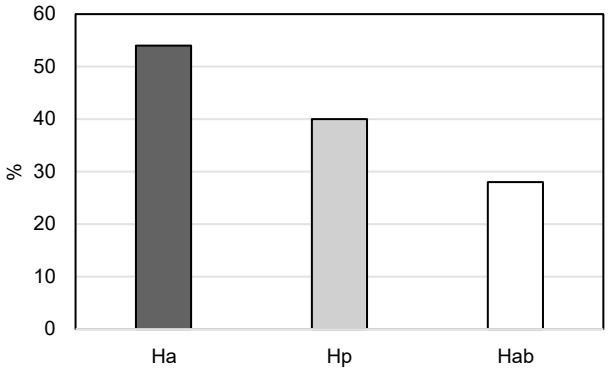


**Fig. 1.** Percentage decrease of *H. annosum* (Ha), *H. parviporum* (Hp) and *H. abietinum* (Hab) culture diameters under the influence of *P. gigantea* metabolites at 7 days after inoculation
**Rys. 1.** Procentowe zmniejszenie średnicy kultur *H. annosum* (Ha), *H. parviporum* (Hp) i *H. abietinum* (Hab) pod wpływem metabolitów *P. gigantea* po 7 dniach od inokulacji

The strongest reduction was found in *H. annosum* 5/2 (61%). In turn, the smallest reduction of mycelium growth in *H. annosum* isolates was caused by *P. gigantea* PgS (mean 37% in relation to the control; Fig. 2, Table 3). All *H. annosum* isolates under the influence of metabolites of *P. gigantea* isolates developed significantly more slowly ($p < 0.0001$) compared to the control. Mycelium of *H. parviporum* growing in the presence of *P. gigantea* metabolites was by 8% up to 63% smaller than the control, while the weakest effect of metabolites on the pathogen mycelium was recorded for isolate PgS (Fig. 2, Table 3). Mycelium growth in *H. parviporum* Suwałki I was reduced the strongest

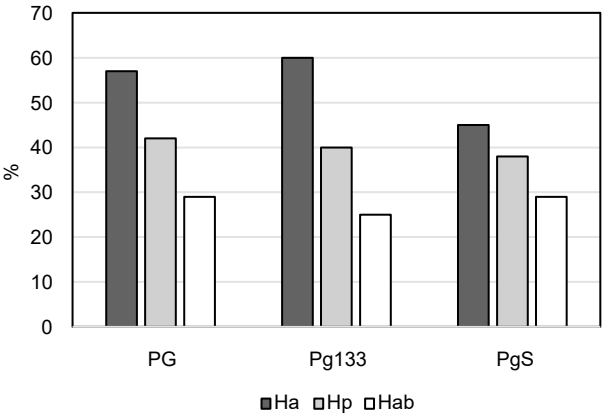


**Fig. 2.** Percentage decrease of culture diameter in *H. annosum* (Ha), *H. parviporum* (Hp) and *H. abietinum* (Hab) under the influence of metabolites of individual *P. gigantea* isolates (PG, PG133, PgS) at 7 days after inoculation
**Rys. 2.** Procentowe zmniejszenie średnicy kultur *H. annosum* (Ha), *H. parviporum* (Hp) i *H. abietinum* (Hab) pod wpływem metabolitów poszczególnych izolatów *P. gigantea* (PG, PG133, PgS) po 7 dniach od inokulacji

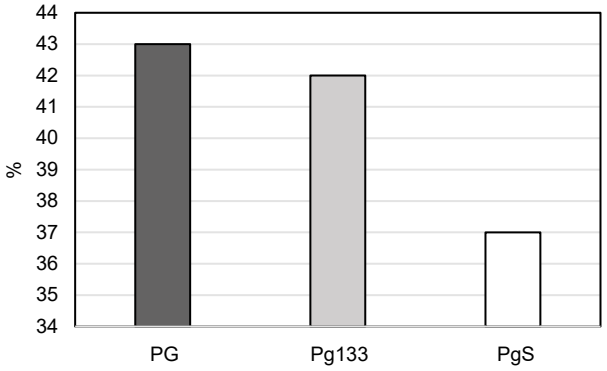


**Fig. 3.** Percentage decrease of *Heterobasidion* culture diameter under the influence of metabolites of individual *P. gigantea* isolates (PG, PG133, PgS) at 7 days after inoculation
**Rys. 3.** Procentowe zmniejszenie średnicy kultur grzybów rodzaju *Heterobasidion* pod wpływem metabolitów poszczególnych izolatów *P. gigantea* (PG, PG133, PgS) po 7 dniach od inokulacji

by the saprotroph metabolites (55%). Among the tested *H. parviporum* isolates only Suwałki I did not respond by slowed growth in the presence of metabolites of *P. gigantea* PG ($p > 0.07212$) and *P. gigantea*

**Table 3.** Statistically homogeneous groups in terms of mycelium diameter in *Heterobasidion* growing in the presence of *P. gigantea* metabolites as the percentage of the control mycelium after 7 days from inoculation
**Tabela 3.** Grupy statystycznie jednorodne średnicy grzybni *Heterobasidion* rosnących w obecności metabolitów *P. gigantea* jako procent grzybni kontrolnych po 7 dniach od wyszczepiania

| Experimental variant<br>Kombinacja doświadczenia | Percentage of the control<br>Procent kontroli | Statistically homogeneous groups<br>Grupy statystycznie jednorodne | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 11 | 22 | 33 | 44 | 55 | 66 | 77 | 88 |
| PG – *H. annosum* 5/2 | 32.9 | **** | | | | | | | |
| Pg133/10/3 – *H. annosum* 5/2 | 36.5 | **** | **** | | | | | | |
| Pg133/10/3 – *H. parviporum* Suwałki I | 37.5 | **** | **** | | | | | | |
| Pg133/10/3 – *H. annosum* 3/1/1/3 | 38.4 | **** | **** | | | | | | |
| PG – *H. parviporum* Suwałki I | 42.2 | **** | **** | **** | | | | | |
| PG – *H. annosum* 3/1/1/3 | 43.2 | **** | **** | **** | | | | | |
| Pg133/10/3 – *H. annosum* 03/7 | 45.1 | **** | **** | **** | | | | | |
| PG – *H. parviporum* Suwałki II | 46.0 | **** | **** | **** | | | | | |
| PgS – *H. annosum* 5/2 | 46.7 | **** | **** | **** | **** | | | | |
| Pg133/10/3 – *H. parviporum* Suwałki II | 50.8 | **** | **** | **** | **** | **** | | | |
| PG – *H. annosum* 03/7 | 52.5 | **** | **** | **** | **** | **** | | | |
| PgS – *H. parviporum* Suwałki II | 55.7 | **** | **** | **** | **** | **** | **** | | |
| PgS – *H. parviporum* Suwałki I | 57.2 | **** | **** | **** | **** | **** | **** | | |
| PgS – *H. annosum* 03/7 | 57.9 | **** | **** | **** | **** | **** | **** | | |
| PG – *H. abietinum* 96070 | 59.5 | **** | **** | **** | **** | **** | **** | | |
| PgS – *H. annosum* 3/1/1/3 | 61.6 | **** | **** | **** | **** | **** | **** | | |
| PG – *H. abietinum* 17021A | 64.9 | **** | **** | **** | **** | **** | **** | **** | |
| Pg133/10/3 – *H. abietinum* 17021A | 64.9 | **** | **** | **** | **** | **** | **** | **** | |
| PgS – *H. abietinum* 17021A | 66.7 | | **** | **** | **** | **** | **** | **** | |
| PgS – *H. abietinum* 96070 | 71.7 | | | **** | **** | **** | **** | **** | **** |
| PgS – *H. parviporum* 17004 | 73.3 | | | **** | **** | **** | **** | **** | **** |
| Pg133/10/3 – *H. abietinum* 20056 | 75.0 | | | | **** | **** | **** | **** | **** |
| PG – *H. abietinum* 20056 | 75.2 | | | | | **** | **** | **** | **** |
| Pg133/10/3 – *H. abietinum* 96070 | 84.4 | | | | | | **** | **** | **** |
| PG – *H. parviporum*17004 | 84.8 | | | | | | **** | **** | **** |
| PgS – *H. abietinum* 20056 | 85.9 | | | | | | | **** | **** |
| Pg133/10/3 – *H. parviporum* 17004 | 91.8 | | | | | | | | **** |

PgS ($p > 0.3759$). In turn, mycelium of *H. abietinum* isolates was inhibited by *P. gigantea* metabolites by 14–41%, with *H. abietinum* 20056 being least susceptible to the inhibitory effect of the saprotroph metabolites (21%; Fig. 2, Table 3). Metabolites of the tested *P. gigantea* isolates had no significant effect on mycelium growth in *H. abietinum* 20056 ($p > 0.6347$), while metabolites of *P. gigantea* Pg133/10/3 did not influence mycelium growth in *H. abietinum* 17004 ($p > 0.2134$). In turn, mycelium of *H. abietinum* 17021A in the presence of metabolites of the *P. gigantea* isolates developed significantly more slowly ($p < 0.0001$). The effect of metabolites of *P. gigantea* PG on all the *Heterobasidion* isolates was the strongest, similarly as in the case of *P. gigantea* Pg133, whereas the weakest effect was found for *P. gigantea* PgS (Fig. 3, Table 3).

## DISCUSSION

Secondary metabolites are by-products formed in fungi in the course of metabolism. Some of them protect fungi against other microorganisms or repel parasites. In turn, others promote their development following the host's death. Another function of metabolites is also connected with their action stimulating or reducing pathogenicity of fungi (Oduro et al., 1976; Sonnenbichler et al., 1989; 1993; 1997; Vey et al., 2001 after Łakomy, 2004).

The tested *P. gigantea* isolates denoted as PG and PG133 had a comparably strong effect on *Heterobasidion* isolates, in contrast to PGS. However, when analysing the influence of *P. gigantea* metabolites on individual *Heterobasidion* isolates it may be stated that these reactions were related to the properties of individual pathogen isolates within each species. For example, metabolites of *P. gigantea* PG133 inhibited mycelium growth of *H. parviporum* 17004 by 8.1%, while in *H. parviporum* Suwałki I it was by 62%. Metabolites of that isolate exhibited the strongest effect on *H. annosum* isolates, because they reduced their growth by 55.6–75% in relation to the control. The strongest reducing influence of metabolites produced by *P. gigantea* isolates was recorded for *H. annosum* 5/2 (reduction of the culture by 64.3–74.1%), whereas it was weakest in the case of *H. parviporum* 17004 (8.2–22.7%).

Many fungi exhibit antagonistic properties in relation to *H. annosum* due to the metabolites released to the substrate. Sierota (1976) showed that antibiotics produced by *T. viride* Pers. within a short time inhibited mycelium growth of the pathogen. Vey et al. (2001 after Łakomy, 2004) investigated the action of certain metabolites released by fungi. They stated that harmful substances or those inhibiting fungal growth may be produced e.g. by *T. harzianum* (gliotoxins, hydrolitic enzymes, tricholin). In turn, metabolites of other fungal species may be toxic for plants causing their diseases and reducing their growth. Similarly, effects of the influence of metabolites were presented in studies on the influence of the soil medium community on mycelium growth of pathogens, including *H. annosum.* In research concerning the interactions between mycelia of soil fungi and the pathogen the inhibitory zone free from fungal hyphae was reported, resulting from the action of metabolites in this substrate zone (Mańka et al., 1993; Mańka and Łakomy, 1995a; 1995b). Results of *in vitro* studies also indicate that mycelium growth of *H. annosum* isolates may be limited in various ways by saprotrophic fungi (Łakomy et al., 1998; Werner et al., 1995). Among these fungi tested in dual cultures the effect of various *P. gigantea* isolates inhibited mycelium growth in 33 *H. annosum* isolates by 3% up to 81%. In that study growth of *P. gigantea* was also limited by *H. annosum* by 1% to 36% (Łakomy et al., 1998).

Many authors investigated the influence of metabolites on fungal growth (Łakomy, 2004; Sonnenbichler et al., 1997; Szwajkowska-Michałek et al., 2018; Raziq, 2000). Łakomy (2004) stated that almost all saprotroph isolates tested in his study produced secondary metabolites, which limited *Armillaria* growth. In this way they reduced mycelium mass in the pathogen isolates by as much as over 90% compared to the control. This was recorded for isolates of *Bjerkandera adusta* (Willd) P. Karst, *Hypholoma fasciculare*, *H. sublateritium* (Schaeff.) Quél., *Kuehneromyces mutabilis* (Schaeff.) Singeli A. H. Sm. and *Pleurotus ostreatus* (Jacy) P. Kumm. *Armillaria* species exhibiting the weakest response to the presence of metabolites of the above-mentioned saprotrophic fungi included *A. borealis* Marxm. et Korhonen, *A. cepistipes* Velen. and *A. mellea* (Vahl) P. Kumm. It was shown that metabolites produced by fungi under laboratory conditions are not always produced by a given species in the natural environment. In some cases they may be secreted, but are rapidly degraded under the influence of various factors (Raziq,

2000). Sonnenbichler et al. (1997) showed that toxic metabolites produced by *Armillaria* were degraded soon after being formed. Subsequently these fungi produced other metabolites, which were not toxic. Szwajkowska-Michałek et al. (2018) determined the profile of volatile metabolites produced by isolates of *Trametes versicolor.* Studies were conducted on isolates with known antagonistic properties in relation to various *Armillaria* species. Those authors stated that the profiles belonging to two different isolates vary in terms of the number and quality of produced compounds. Tested *T. versicolor* isolates differed in terms of the presence and quantities of fungistatic compounds such as heptanal and butanal, which were produced by both isolates, as well as 2-methylbutanal, which was produced by only one of them. According to those authors, not all isolates of the same saprotrophic fungus species will constitute a homogeneous barrier and an element of resistance of the natural environment against *Armillaria* spp.

Based on this study it may be stated that the response of the pathogen isolates to metabolites of individual saprotroph isolates differs depending on the specific properties of the tested organisms – both pathogens and isolates. Neither was a particularly susceptible pathogen isolate identified, nor was a saprotroph isolate distinguished, which metabolites would exhibit a strong effect on all the tested pathogen isolates.

When using fungi in biological control treatments many characteristics of the proposed isolates need to be considered, such as e.g. their capacity to produce toxic metabolites for the pathogens, against which they will be used. It will be crucial to characterise such a capacity when selecting a saprotroph isolate for the production of a specific biopreparation.

## CONCLUSIONS

Secondary metabolites of *Phlebiopsis gigantea* isolates inhibit mycelium growth in *Heterobasidion* spp. *in vitro*. The degree of such a reduction was varied.

Response of the pathogens to metabolites of individual saprotroph isolates depended on the specific properties of an individual *Heterobasidion* isolate.

*Heterobasidion annosum* and *H. parviporum* turned out to be the most sensitive species to the action of *Phlebiopsis gigantea* metabolites, while *H. abietinum* was least sensitive.

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## WPŁYW MIESZANINY METABOLITÓW *PHLEBIOPSIS GIGANTEA* (FR.) JÜLICH NA WZROST GRZYBNI *HETEROBASIDION* SPP. *IN VITRO*

### ABSTRAKT

**Wstęp.** Ochrona biologiczna przed hubą korzeni wykorzystuje naturalne zdolności antagonistyczne grzyba saprotroficznego *Phlebiopsis gigantea* (Fr.) Jülich.

**Celem badań** było określenie wpływu metabolitów *P. gigantea* na wzrost grzybni trzech występujących w Polsce gatunków *Heterobasidion*.

**Metodyka.** Do badań użyto dziewięć izolatów *Heterobasidion* oraz trzy izolaty *P. gigantea.*

**Wyniki.** Metabolity wtórne saprotrofa najsilniej wpływały na ograniczanie wzrostu grzybni *H. annosum* i *H. parviporum*, natomiast najsłabiej na grzybnię *H. abietinum*. Analiza wariancji wykazała istotne różnice pomiędzy średnią wielkością grzybni w poszczególnych kombinacjach doświadczenia ($P < 0{,}00001$), a na podstawie testu Tukeya można wyróżnić osiem grup homogenicznych oddziaływania metabolitów izolatów *P. gigantea* na poszczególne izolaty *Heterobasidion*.

**Wnioski.** Stwierdzono, że metabolity wtórne izolatów *P. gigantea* w stopniu zróżnicowanym ograniczają wzrost grzybni *Heterobasidion* spp. *in vitro*.

**Słowa kluczowe:** *Phlebiopsis gigantea*, *Heterobasidion* spp., metabolity wtórne, ochrona biologiczna