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## Growth of two blue-stain fungi associated with *Tetropium* beetles in the presence of callus cultures of *Picea abies*

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**Abstract:** The callus tissue can be used to evaluate the potential ability of microorganisms to cause disease. The blue-stain fungi, *Grosmannia piceiperda* and *Ophiostoma tetropii* are important associates of *Tetropium* spp. in Poland. The opinions about their virulence are controversial. Here, we examined the growth mycelium of the *G. piceiperda* and *O. tetropii* in presence of the non-embryogenic cultures of Norway spruce, and accumulation of soluble and insoluble proteins in this callus.

The growth mycelium of one isolate of *G. piceiperda* was significantly stimulated whilst another isolate of the fungus and both isolates of *O. tetropii* were unaffected by the presence of the callus. The significant higher ( $P < 0.05$ ) amount of soluble protein, was noted in the callus with both isolates of *G. piceiperda*. In contrast to *G. piceiperda*, the callus with *O. tetropii* had a similar concentration of soluble protein as the control. The importance of these results with respect to the pathogenic abilities and the *in vivo* behaviour of the examined fungi is discussed.

**Additional key words:** callus, dual cultures, *Grosmannia piceiperda*, Norway spruce, *Ophiostoma tetropii*

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### Introduction

Most subcorticolous insects introduce various microfungi to their host trees. Blue-stain fungi and *Geosmithia* spp. are frequent associates of phloem-feeding insects infesting trees (Kirisits 2004; Kolařík et al. 2008).

The blue-stain fungi, *Grosmannia piceiperda* (Rumbold) Goid. and *Ophiostoma tetropii* Math.-Käärik are consistent associates of *Tetropium* spp. in Poland (Jankowiak and Kolařík 2010). The results of some inoculation experiments indicated that *G. piceiperda* was pathogenic for mature trees (Harding 1989; Solheim 1993; Sallé et al. 2005; Jankowiak et al. 2009). Virulence of some isolates of *G. piceiperda* towards Norway

spruce seedlings has also been reported by Jankowiak and Kolařík (2010). On the other hand, in Kirisits' studies (1998) this species was found to be weakly pathogenic. The true pathogenicity of *O. tetropii* is also uncertain. Some isolates were non-pathogenic towards Norway spruce seedlings (Jankowiak et al. 2009; Jankowiak and Kolařík 2010; WWW document; <http://cfs.nrcan.gc.ca/index/summarybslb>), whereas others play an important role in the death of spruce trees (Sallé et al. 2005).

The pathogenicity of blue-stain fungi has been most often studied with the use of fungal inoculation of large trees and seedlings (Kirisits 2004). Recently the callus tissue has also been used for the *in vitro* estimation of resistance of forest trees to fungal patho-

gens (e.g. Kvaalen et al. 2001). The reaction of the fungus with respect to the embryogenic or non-embryogenic callus may constitute a reflection of this fungus' virulence in natural conditions (Hřib et al. 1995; Niemi et al. 1998; Kvaalen and Solheim 2000; Vookova et al. 2006).

In higher plants, biotic stress (e.g. pathogen infection) as well as abiotic stress (light, temperature, water and salinity) often induce the synthesis and accumulation of many proteins, including pathogenesis-related (PR) proteins. Some of these proteins are hydrolytic and can degrade components of invasive pathogens (Kärenlampi et al. 1994; Roberts et al. 2002). The accumulation of storage protein, including defence-related proteins, is a major change that occurs during the growth of embryos in the presence of fungal pathogens (Vookova 2006).

The objective of the study was to examine 1) the growth mycelium of the *G. piceiperda* and *O. tetropii* isolates which were before used in the virulence experiments (Jankowiak et al. 2009; Jankowiak and Kolařik 2010), in the presence of the callus culture of Norway spruce, 2) protein accumulation in callus cultures of spruce in the presence of *G. piceiperda* and *O. tetropii* isolates.

## Materials and methods

### Plant materials

The non-embryogenic callus (NEC) of Norway spruce [*Picea abies* (L.) Karst.] was initiated on mature zygotic embryos isolated from seeds originating from the Bieszczadzki National Park. The initiation of the callus on the embryos was performed *in vitro* on the BM-3 medium (Gupta and Durzan 1987) with the addition of 2.2  $\mu\text{M}/\text{dm}^3$  benzylaminopurine (BA) and

1.0  $\text{g}/\text{dm}^3$  casein hydrolysate as well as 10.0  $\text{g}/\text{dm}^3$  sucrose. The callus was proliferated on the BM-3 medium with the same composition and with the addition of 1.1  $\mu\text{M}/\text{dm}^3$  BA and 9.0  $\mu\text{M}/\text{dm}^3$  2,4-D. The callus cultures were grown on Petri dishes in the dark, at the temperature of 25°C for 4 months. The callus was passaged every two weeks.

### Fungal isolates

The isolates of *G. piceiperda* (no. 178/RJ and 335/RJ) and *O. tetropii* (no. 283/RJ and 319/RJ) were obtained in 2006 from *Tetropium* beetles, and then were deposited in the culture collection of the Laboratory of Department of Forest Pathology, Hugo Kořataj University of Agriculture in Kraków. The fungi grew on Petri dishes on malt agar (2% malt, 2% agar) for 7 days and then were transferred to Petri dishes with the BM-3 medium.

### Interaction between fungi and callus: growth and callus appearance

After 10 days 1 × 1 cm inocula were sampled from fungal cultures grown on BM-3 medium and placed in the centre of a Petri dish, with non-embryogenic callus of Norway spruce with the diameter of 1 cm (0.5 mg).

The callus was situated at the distance of 2.5 cm from the centre of the fungus inoculum and 5 mm from the dish edge. Such an arrangement of this dual-culture made it possible to measure the mycelium growth towards the callus and away from it. The same measurements were taken for the control callus lines and the control fungus isolates. The dual-cultures (four replications of each isolate), and controls for callus and fungal isolates were kept in the dark, at the temperature of 25°C. Measurements of mycelial

Table 1. The Student-test – results to compare the average amounts of proteins (mg/g) generated by the non-embryogenic callus of Norway spruce (*Picea abies*) in dual cultures

Fungus:	Isolates:	<i>G. piceiperda</i>		<i>O. tetropii</i>	
		178	335	283	319
Soluble protein (SP):					
<i>G. piceiperda</i>	178				
	335	0.1794			
<i>O. tetropii</i>	283	0.0368*	0.5728		
	319	0.0111*	0.4774	0.9118	
Control		0.0059*	0.4124	0.8014	0.9930
Insoluble protein (IP):					
<i>G. piceiperda</i>	178				
	335	0.0024*			
<i>O. tetropii</i>	283	0.0103*	0.6465		
	319	0.0004*	0.1818	0.5529	
Control		0.0087*	0.4885	0.9971	0.5596

\* Statistically essential difference on the level  $\alpha = 0.05$ ; probability "p" test hypothesis.

Table 2. The comparison of the average protein contents in the callus (soluble proteins – SP and insoluble proteins IP [mg/g]) in correlation to the growth of the analyzed fungi – Student's t-test

Fungal growth towards the callus	Average SP [mg/g]	Standard deviation SP (mg/g)	Average IP [mg/g]	Standard deviation IP (mg/g)
Rapidly growing mycelium ( <i>G. piceiperda</i> )	0.9325	0.4721	0.4721	0.2986
Slowly growing mycelium ( <i>O. tetropii</i> )	0.7747	0.2023	0.2986	0.1906
control	0.5148	0.1675	0.4802	0.2784
Results of Student's t-test comparing the averages	t = 1.918; df = 13; p = 0.0773		t = 2.523; df = 13; p = 0.0254 statistically significant differences	

growth compared to the callus were performed every 24 hours for 10 days of the dual culture. The distance between the hyphae and callus was measured with a ruler at the bottom of a Petri dish. At that time phenotypic changes of the callus and the fungus were also macro- and microscopic observed, and they are described in the "Results" section.

### Protein analysis

Soluble proteins were extracted from about 100 mg of the callus in 1 ml of Tris-HCl buffer (50 mM Tris [tris(hydroxymethyl)-aminomethane]-HCL, pH 7.5, 1mM EDTA, pH 8, 10 mM MgCl<sub>2</sub>, 14.0 mM [v/v] b-mercaptoethanol). Samples were centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was used for determination of protein content and pellets were incubated for 24 h at room temperature in Tris buffer containing 0.1% SDS to extract insoluble proteins (Hashimoto et al. 1989). Protein levels were estimated by the Bradford method (1976) using bovine serum albumin as the standard.

### Statistical analysis

The differences between the length of the mycelium radius of studied fungal isolates were examined using the Friedman ANOVA test. The analysis was impossible to apply due to the lack of convergence of the radius lengths with theoretical normal distribution. Uniform groups were distinguished by means of the HSD Tukey test. A comparison was also made between pairs of the radii of particular fungus mycelia with the corresponding control mycelia. The non-parametric Wilcoxon test of matched pairs was used for this purpose. Average soluble protein (SP [mg/g]) and insoluble protein (IP [mg/g]) content produced by the callus was compared. Student's t-test at significance level of 0.05 was applied to examine the relation of the mycelium growth and the amount of proteins produced by the callus (Table 2). All analyses were performed with the use of the STATISTICA programme, version 8.0.

### Results

In the dual-culture experiment, callus tissue significantly stimulated the mycelium growth of *G.*

*piceiperda* isolate 335/RJ (Fig. 1). Another isolate of this fungus and also isolates of *O. tetropii* showed similar length of mycelium radius growth both towards the callus and from it as compared to the controls (Fig. 1). After 8 days from inoculation the following phenotypic changes were observed in the callus overgrown by the fungus *G. piceiperda*: the callus became dark-brown, dehydrated, compacted and it eventually died. White fungal hyphae completely overgrew the callus and even penetrated into the callus cells, preventing its further development (Fig. 3). On the other hand, Norway spruce callus in the dual culture with *O. tetropii* was much brighter (light brown). Fragments of its tissue remained alive, which was confirmed by microscopic observations. White, compacted fungal hyphae of *O. tetropii*, forming stromatic structure, did not overgrow the callus – it only spread on a medium (Fig. 3). In the dual-culture with *G. piceiperda* and *O. tetropii*, the NEC showed variable protein concentration as compared to the control (Fig. 2). In NEC inoculated with the fungi and in the control, more soluble protein was detected. The significantly ( $P < 0.05$ ) higher amount of soluble protein, in comparison with control, was only found in NEC with using isolates of *G. piceiperda*. The NEC with isolate 178/RJ had considerably more protein than the NEC with isolate 335/RJ. In contrast to *G. piceiperda*, the callus with *O. tetropii* possessed similar concentration of soluble protein as the control. The Student-test results for dependent variable on the significance level  $\alpha = 0.05$ , indicates, that in the case of soluble proteins, the two tested fungal isolates of *O. tetropii*, differ statistically from the isolate 178 of *G. piceiperda*. In the case, however of insoluble proteins, the isolate nr 178 fungus *G. piceiperda* significantly differs from all tested isolates (Table 1). Student's t-test applied to examine the relationship between the mycelial growth and the amount of proteins produced by the callus showed that the average content of soluble protein – SP (mg/g) and insoluble protein – IP (mg/g) in the callus samples, towards which the mycelium grew rapidly, is higher than in the samples, towards which the mycelium grew slowly. However, in the case of insoluble proteins (IP) this statistical difference was at significance level of 0.05 (Student's t-test: t = 2.523, df = 13, p = 0.0254) (Table 2).

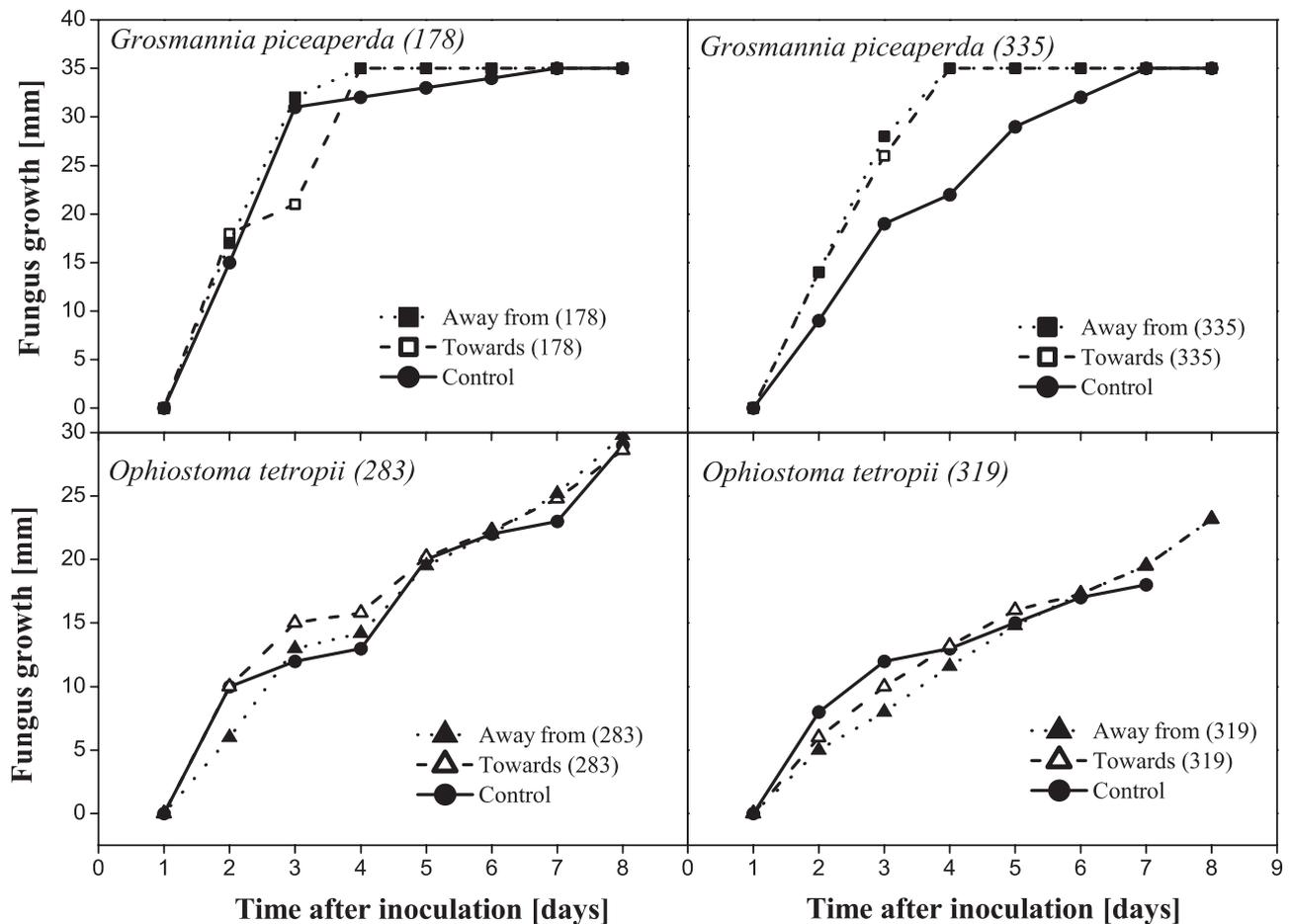


Fig. 1. Growth of *Grosmannia piceaperda* (isolates 178 and 335) and *Ophiostoma tetropii* (isolates 283 and 319) away from and towards callus of the *Picea abies* after 8 days of cultivation. The control is fungus inoculated in dishes without callus

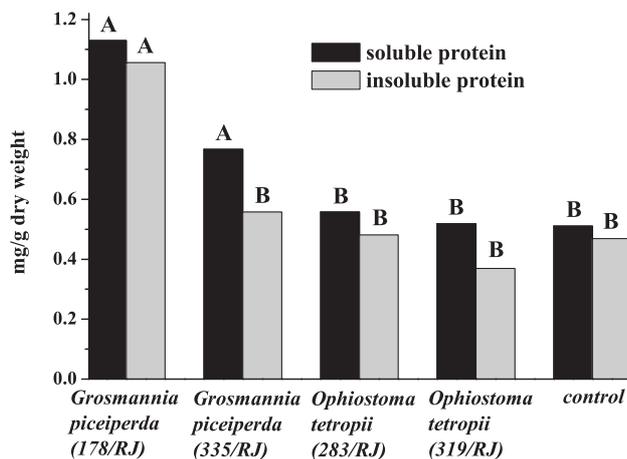


Fig. 2. Soluble and insoluble protein levels in calli after 72 h cultivation with isolates of *Grosmannia piceaperda* and *Ophiostoma tetropii* (different letters represents statistical differences ( $P < 0.05$ ) within proteins types)

## Discussion

The interaction between fungi and conifer callus has been studied by several authors (Hřib and Rypacek 1978; Woodward and Pearce 1988; Ragazzi et al. 1995; Sirenberg et al. 1995; Kvaalen and Solheim 2000; Vookova et al. 2006; Nawrot-Chorabik and Jankowiak 2010). Czech and Norwegian authors (Hřib and Rypacek 1978), Kvaalen and Solheim 2000) found an effect of the *P. abies* callus on the growth of *H. annosum*, especially during its initial growth. A similar reaction has been displayed for blue stain fungus, *Ceratocystis polonica* (Kvaalen and Solheim 2000) and for wood-destroying fungus, *Phaeolus schweinitzii* (Fr.) Pat. (Vookova et al. 2006). These studies show that pathogenic fungi or regarded as primary invaders in tree sapwood are stimulated by callus and show an ability to colonize it, while saprotrophes recognized as subsequent colonizers of tree sapwood are inhibited by callus (Hřib and Rypáček 1981; Hendry et al. 1993; Nawrot-Chorabik and Jankowiak 2010). However, in contrast to these studies, Sirenberg et al. (1995) and Woodward and Pearce (1988) recorded no effect of the callus of *Picea* spp. on the growth of *H. annosum*.



Fig. 3. Appearance of the non-embryogenic callus of *Picea abies* after 8 days inoculation with: A – *Grosmannia piceiperda*, the arrow – colonized and dead callus; B – *Ophiostoma tetropii*, the arrow – non-colonized and “healthy-look” callus

In our experiments significant differences were observed only in the mycelial growth of one isolate of *G. piceiperda* in the presence of spruce callus (four days after inoculation). The stimulatory effect of spruce callus was also evident in another isolate of *G. piceiperda* but only 4 days after inoculation. According to earlier observations of tree callus (Hendry et al. 1993; Kvaalen and Solheim 2000; Vookova et al. 2006; Nawrot-Chorabik and Jankowiak 2010), this fact may indicate that the tested *G. piceiperda* isolates may cause the disease.

The stimulation of the mycelium growth of *G. piceiperda* by callus tissues was also connected with the protein content. A high amount of soluble protein in NEC inoculated with *G. piceiperda* isolates suggests they are virulent towards *P. abies*. The callus of spruce probably synthesized a new defence protein in response to the presence of the *G. piceiperda*. Our results regarding virulence of *G. piceiperda* isolates correspond or contradict with the one's noted by some authors in vivo experiment (Harding 1986; Kirisits 1998; Solheim 1993; Sallé et al. 2005; Jankowiak et al. 2009). Recently, Jankowiak and Kolařík (2010) have showed that the inoculated seedling of *P. abies* were severely affected by isolate 178/RJ of *G. piceiperda*. Of the tested two isolates of this species, 178/RJ produced higher amount of protein than 335/RJ. This difference between isolates may be connected with their variability in virulence. Such variation has been also reported for isolates of *Leptographium wingfieldii* M. Morelet (Lieutier et al. 2004) and *C. polonica* (Krokene and Solheim 1998, 2001).

In this *in vitro* experiment, the similarity of mycelium growth rates of both isolates of *O. tetropii* in the presence and absence of spruce callus suggested that there was little or no effect of callus on this fungus. This result is well corresponded with the amount of soluble protein in *P. abies* callus. The lack of a defen-

sive response of callus to the presence of *O. tetropii* is in agreement with inoculation experiments using the same fungal isolates (Jankowiak and Kolařík 2010; Jankowiak et al. 2009).

In conclusion, our *in vitro* results have confirmed that studied *G. piceiperda* isolates are virulent while *O. tetropii* appears to be non-virulent to Norway spruce. Differences in the ability to cause disease of *G. piceiperda* and *O. tetropii* obtained in many inoculation experiments (Harding 1989; Solheim 1993; Kirisits 1998; Sallé et al. 2005; Jankowiak et al. 2009; Jankowiak and Kolařík 2010) could result from the different inoculation techniques and virulence variability of isolates. The present study has demonstrated that the virulence of the isolates may be related to the content of soluble protein in the host callus. The performed statistical analyses comparing the average protein contents in the callus indicated the relationship between the amount of proteins produced and the mycelial growth rate, and – in consequence – its virulence (Table 2). It appears that this method can be very helpful in estimating phytopathogenic properties of fungi.

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