University of Agriculture in Cracow, Cracow, Poland

CONTRIBUTION TO PATHOGENICITY OF THREE BLUE-STAIN FUNGI ASSOCIATED WITH THE PINE SAWYER BEETLE (*MONOCHAMUS GALLOPROVINCIALIS*) (COLEOPTERA: CERAMBYCIDAE) TO SCOTS PINE IN POLAND[1]

R. Jankowiak, R. Rossa and P. Bilański

Abstract

Scots pine (*Pinus sylvestris*) trees were inoculated with three fungi (*Ophiostoma minus*, *O. piceae* and *Leptographium procerum*) associated with the pine sawyer beetle (*Monochamus galloprovincialis*) or with sterile medium (control) to evaluate their pathogenicity. Inoculation densities were 400 and 800 inoculation points per 1 m^2 in a 60 cm wide band at breast height. The inoculated trees were examined in October 2007, 23 weeks after inoculation. None of the inoculated trees was dead. Inoculation with *O. minus* produced significantly largest lesions. The amount of occluded sapwood was also greater following *O. minus* inoculations than other fungi or the control. Also *L. procerum* caused significantly longer necrotic lesions and more occlusions in the sapwood than *O. piceae* and the controls.

Key words: blue-stain fungi, *Monochamus galloprovincialis*, pathogenicity, *Pinus sylvestris*

Introduction

Blue-stain fungi, also known as ophiostomatoid fungi, are commonly associated with phloeophagous bark beetles. These microorganisms are an important fungal group as they cause blue-staining in freshly cut logs, and some of them are capable of killing mature trees (Kirisits 2004). Beside bark beetles also other arthropods may carry their spores. Among them there are forest cerambycid beetles

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which are considered secondary pests of trees (Mathiesen 1950, Kotýnková-Sychrová 1966, Wingfield 1987, Jacobs et al. 2000, Jacobs and Kirisits 2003, Jacobs et al. 2003, Jankowiak and Rossa 2007, Jankowiak and Kolařík unpublished). However, some species are able to kill healthy trees and act as primary forest pests. *Monochamus galloprovincialis* usually colonizes dying or stressed *Pinus sylvestris* trees, but when the population level of *M. galloprovincialis* is high it may also attack healthy trees (Dominik and Starzyk 1989).

Little is known about blue-stain fungi associated with *Monochamus* species. Jacobs et al. (2000) reported that *Leptographium sibiricum* and *Ophiostoma* species were carried by *M. urussovi* on Siberian fir. Wingfield (1987) found a few *Ophiostoma* species in the galleries of *Monochamus* species. In a recent study in Poland, seven blue-stain fungi, including *Graphium pseudormiticum*, *G. pycnocephalum*, *Graphium* sp.'W', *L. procerum*, *O. minus*, *O. piceae* and *O. piliferum* were isolated from the *M. galloprovincialis* beetles and their galleries (Jankowiak and Rossa 2007). Among these, *O. minus* is the primary fungus during sapwood colonization after *Tomicus piniperda* attack (Jankowiak and Kurek 2006) and it is the most pathogenic one when inoculated into large *P. sylvestris* trees (Lieutier et al. 1989, Solheim and Långström 1991, Solheim et al. 1993, 2001). In Polish inoculation experiment carried out by Jankowiak (2006) the species had also displayed pathogenicity to *P. sylvestris* seedlings. However, neither of ophiostomatoid species have yet been evaluated as a pathogen of Scots pine by inoculating large trees in Poland. The knowledge on pathogenicity of ophiostomatoid fungi isolated from *M. galloprovincialis* is also very limited.

The purpose of this preliminary study was to evaluate the pathogenicity of *O. minus*, *O. piceae* and *L. procerum* (the most important associates of *M. galloprovincialis* in Poland) to large Scots pine trees.

Materials and methods

Inoculations were performed on Scots pines at Mielec-Mościska, Mielec Forest District (50°19'25" N, 21°29'39" E). Eight trees (55 years old) were selected on the basis of similarity in general appearance and size. The trees were "healthy-looking" with a mean diameter at breast height (DBH) of 11.7 (10.5–14.5) cm. Each tree was assigned randomly to one of eight treatments (one tree per treatment). The treatments were inoculation with *O. minus* (O.m./545), *O. piceae* (O.p./518), *L. procerum* (L.p./544) at 400 or 800 inoculation points per 1 m^2 of bark surface in a 60-cm-broad band at breast height. Thus, the numbers of inoculation points ranged from 60 to 144 per tree. Inoculation with sterile 2% malt agar (MEA, agar 20 g/l, malt 20 g/l) was used as control. Inoculation densities were selected basing on previous pathogenicity tests with similar blue-stain fungi (Solheim et al. 1993, Långström et al. 2001, Solheim et al. 2001).

The trees were inoculated on 9 May 2007 with two-week-old mycelium grown on 2% MEA or with sterile MEA. The isolates of fungi were obtained at Mie-

lec-Mościska in July 2006 from *M. galloprovincialis* adults and their galleries. Fungi isolates are maintained at the University of Agriculture in Cracow, Poland.

Bark plugs were removed with a 5-mm cork borer up to the cambium zone. Small pieces of fresh growing mycelium were inserted into the holes and the bark plugs were put back. In control trees bark plugs were put back after inserting sterile MEA. The inoculation holes were distributed in horizontal rings located 10 cm away from each other (at 400 inoculation points per 1 m^2) or 5 cm away (at 800 inoculation points per 1 m^2). In each ring the distance between the inoculation points was 5 cm.

The trees were felled on 15 October 2007 and logs (1.2 m long) containing inoculated region were cut and transported to the laboratory. Bark was stripped from the logs within 48 h of harvesting and, as in Kirisits' studies (1998), the extent of dead phloem was estimated visually according to a scale from 1 to 6. The lengths and widths of the lesions on the sapwood surface were measured at each inoculation point. The vertical and lateral extent of every lesion was measured from the outermost discoloured edge on the sapwood surface as the border. Since the lesions between inoculum points overlapped (especially for *O. minus*) lesions lengths were measured only from inoculation points in the top and bottom rows, in a direction away from the main inoculation area. These measurements were doubled to estimate the total lesion length.

Thin discs (about 5 mm) were cut at 10 cm above and below the centre of the inoculation band. The area of occluded sapwood caused by inoculated fungi had resinous appearance and was surrounded by dry, whitish zones. Each disc was photocopied, and healthy and occluded sapwood were determined with image analysis software (ImageJ 1.32j. http://rsb.info.nih.gov/ij/).

Reisolation of fungi was performed at randomly chosen 30 inoculation points on each tree (two pieces of sapwood per inoculation point). Pieces of sapwood were taken from discoloured areas around inoculation points and were disinfected with 96% ethyl alcohol, using cotton wool. The disinfection lasted approximately 15 s and then sapwood samples were dried on filter paper. Samples of sapwood were placed on 2% MEA with 200 mg tetracycline sulphate per 1 l and 100 mg cycloheximide per 1 l to select the *Ophiostoma* and *Leptographium* species. Fungi were identified using morphological characters.

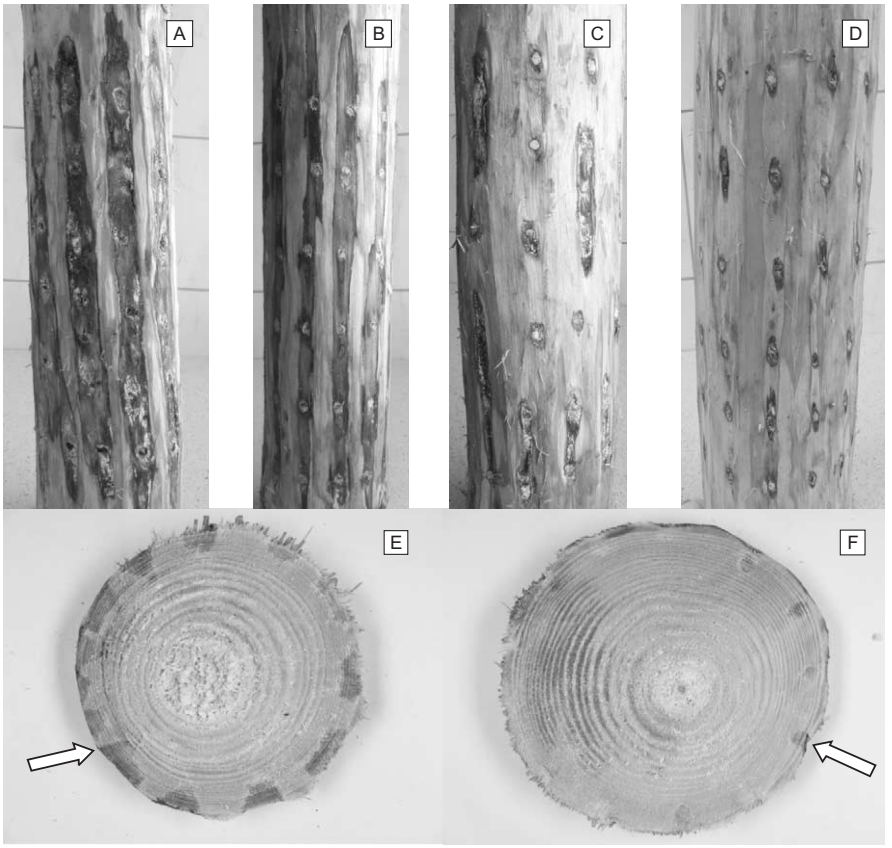
The differences in lesion dimensions among inoculated fungi and control were tested with the nonparametric Kruskal-Wallis ANOVA test implemented in STATISTICA® 8.0 (StatSoft, Inc., USA).

Results

The inoculated fungi were successfully reisolated from 100% of the inoculation points, except *O. piceae*, which was reisolated from 98% of the inoculation points. Neither ophiostomatoid species were isolated from the control trees.

On the basis of foliage condition and colour, no tree showed clear symptoms of dieback at the time of harvest.

All three fungal species tested caused lesions on the sapwood surface, with differences among species. The control lesions and those caused by *O. piceae* were relatively small, whereas *O. minus* and *L. procerum* induced considerably greater lesions (Phot. 1 A–D). Trees inoculated with *O. minus* (400 and 800 inoculation points per 1 m^2) and *L. procerum* (800 inoculation points per 1 m^2) showed long strip lesions along the column of inoculum points on the sapwood (Phot. 1 A, B). The mean length and width of lesions caused by *O. minus* were significantly greater than the control ones and those caused by other fungi. Also *L. procerum* produced longer lesions than the controls and *O. piceae* (Fig. 1 a). However, the fungus caused lesions of similar width as the control ones and those caused by *O. piceae* (Fig. 1 b).



Phot. 1. Lesions on the surface of sapwood caused by *Ophiostoma minus* (A), *Leptographium procerum* (B), *O. piceae* (C) and control (D) at 800 inoculation points per 1 m^2. The lesions were observed 23 weeks after inoculation. Occlusions in sapwood underneath the inoculations points. (E) *O. minus*, 800 inoculation points per 1 m^2. (F) Control, 800 inoculation points per 1 m^2. The arrows indicate the occluded area (photo by R. Jankowiak)

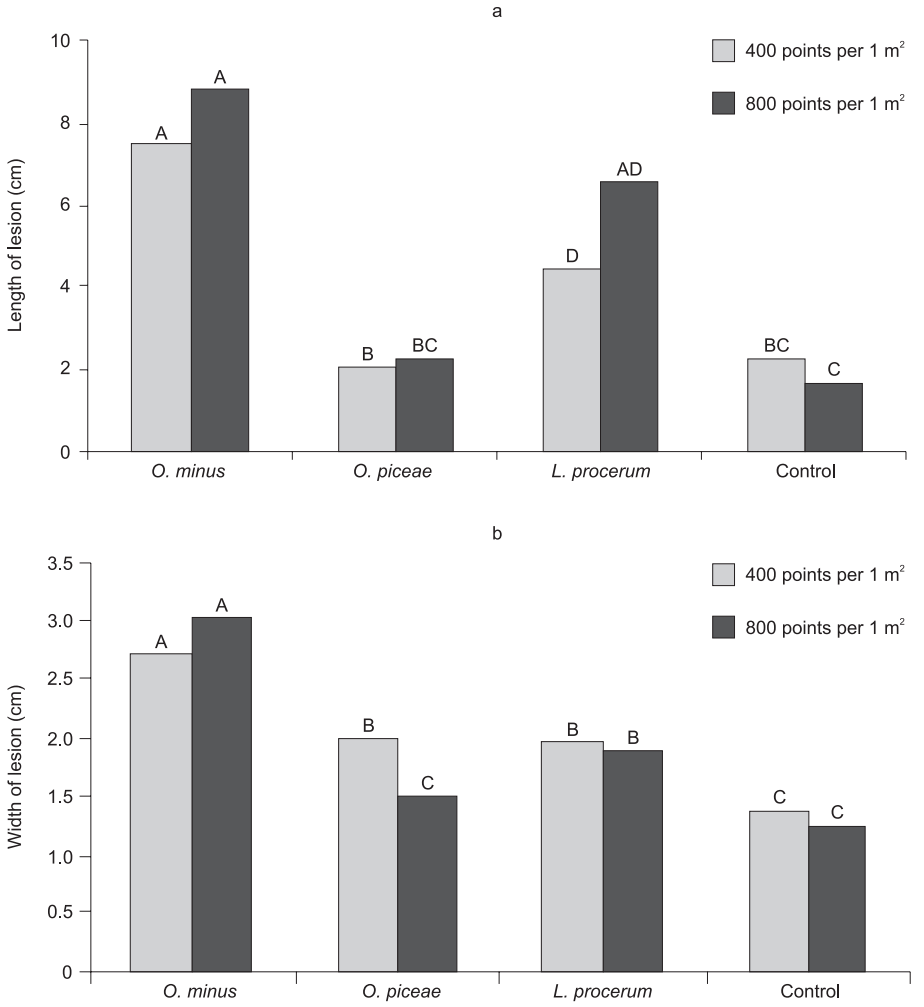


Fig. 1. Mean length of lesions (a) and mean width of lesions (b) on the sapwood of Scots pine inoculated with three blue-stain fungi and control at 400 and 800 inoculation points per 1 m^2. Within each inoculation density, treatments with the same letter were not significantly different (Kruskal-Wallis ANOVA test at p = 0.05)

The fungi in question produced longer lesions at high inoculation density, but the difference was not statistically significant (Fig. 1 a). In contrast to the length of lesions, inoculated fungi, with the exception of *O. minus*, caused wider lesions at low inoculation density. However, these differences were statistically significant only for *O. piceae* (Fig. 1 b).

The amount of phloem killed within the inoculation bands increased with the increasing inoculation density of *O. minus*, *L. procerum* and *O. piceae* (Fig. 2). *Ophiostoma minus* killed more phloem than the other fungi. At the higher inocula-

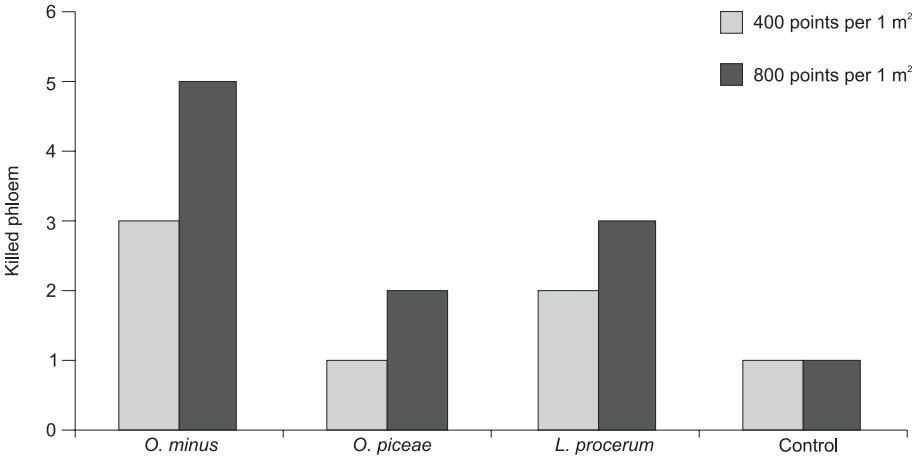


Fig. 2. Killed phloem within inoculation band at 400 and 800 inoculation points per 1 m^2: 1 – small necroses only, 2 – < 20% killed, 3 – 21–40%, 4 – 41–60%, 5 – 60–80%, 6 – > 81% phloem killed

tion density, the fungus killed over 60% of the phloem within the inoculation band (Fig. 2).

Occlusions were observed in the sapwood underneath inoculation points both in the case of the controls and inoculated fungi (Phot. 1 E, F). Sapwood occlusions induced by *O. minus* were significantly greater than occlusions caused by the other fungi and those in control. The amount of occluded sapwood increased with the increasing density of fungal inocula (Fig. 3).

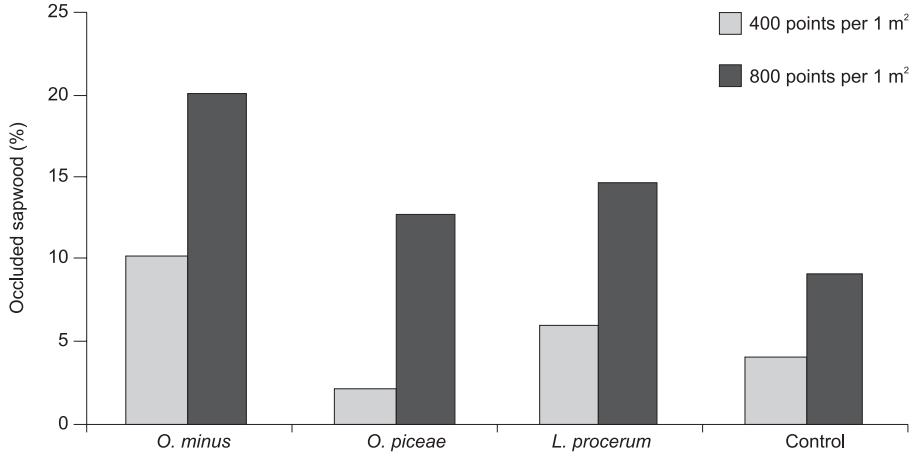


Fig. 3. The amount of occluded sapwood in Scots pine trees following inoculation with *O. minus*, *O. piceae*, *L. procerum* and control at 400 and 800 inoculation points per 1 m^2

Discussion

Three blue-stain fungi associated with *M. galloprovincialis* grew and caused lesions in the Scots pine phloem and sapwood similarly to those previously described in earlier inoculation studies (Långström et al. 1993, Solheim et al. 1993, Långström et al. 2001, Solheim et al. 2001). However, they varied greatly in pathogenicity. *Ophiostoma minus* caused extensive symptoms in Scots pine. It produced long and wide lesions at 400 and 800 inoculation points per 1 m^2, and nearly 75% of the phloem was killed at a inoculation density of 800 points per 1 m^2.

Consistent with previous studies (Långström et al. 1993, Solheim et al. 1993, Långström et al. 2001, Solheim et al. 2001) *O. minus* at high inoculation densities was pathogenic to Scots pine. However, in contrast to earlier observations (Solheim and Långström 1991, Solheim et al. 1993) none of the Scots pine trees was dead or dying 23 weeks after inoculation, but environmental and technical conditions differed (climate, tree diameter, incubation time). In Swedish studies (Solheim et al. 1993) *O. minus* killed significantly smaller Scots pine trees 56 weeks after inoculation. Nevertheless, *O. minus* in this study was able to kill many portions of phloem. It is likely that these trees would have died, providing the harvesting would have been delayed. The pathogenicity of *O. minus* is also in accordance with the results of Jankowiak (2006) who showed pathogenicity of the fungus to Scots pine seedlings. In this study length and width of lesions corresponded well to the sapwood occlusion, indicating that the fungus grew rapidly in the phloem as well as in the sapwood. Långström et al. (2001) reported similar results following inoculation of Scots pine trees in Sweden. Jankowiak and Kurek (2006) also showed that *O. minus* was the first serious colonizer of Scots pine phloem and sapwood infested by *T. piniperda*. These results are also supported by rapid growth of *O. minus* under oxygen-deficient-conditions described by Solheim et al. (2001). Solheim (1991) suggested that the ability to grow under low oxygen levels was characteristic of pathogenic blue-stain fungi.

From among other fungi considered, *L. procerum* seemed to have some pathogenic capability. *Leptographium procerum* caused usually bigger lesions and higher amounts of occluded sapwood than *O. piceae* and control. These results are in agreement with earlier inoculation studies (Wingfield 1986, Harrington and Cobb 1988, Eckhardt et al. 2004, Jankowiak 2006).

Ophiostoma piceae, the major blue-stain fungus associated with *M. galloprovincialis* (Jankowiak and Rossa 2007), was considerably much less pathogenic than *O. minus* and *L. procerum*. This finding is consistent with reports by other authors (Krokene and Solheim 1998, Jankowiak 2006).

Though our preliminary studies showed that *O. minus* was a pathogenic fungus, it seems that this species associated with *M. galloprovincialis* can not play a major role in tree-killing after insect attack. According to the Polish studies (Jankowiak and Rossa 2007) the insect transmits *O. minus* with relatively low frequency. The small degree of association between *O. minus* and *M. galloprovincialis* could not have contributed to the destruction of pine trees defence mechanisms during the attack

of the insect. Similar phenomenon was observed in France between *O. minus* and *T. piniperda* (Lieutier 2004). Isaev et al. (1988) suggested that fungi carried by *M. urussovi* might have played major role in branches' desiccation. Pathogenic *O. minus* carried by *M. galloprovincialis* certainly may also play an important role in branch dieback during maturation feeding, when the beetles damage the bark. The isolation of fungi from branches injured by juvenile beetles could confirm this hypothesis and much research is still needed.

Streszczenie

PRZYCZYNEK DO PATOGENICZNOŚCI TRZECH GRZYBÓW SINIZNOWYCH ZWIĄZANYCH Z ŻERDZIANKĄ SOSNÓWKĄ (*MONOCHAMUS GALLOPROVINCIALIS*) (COLEOPTERA: CERAMBYCIDAE) NA SOŚNIE ZWYCZAJNEJ W POLSCE

Badano patogeniczność wobec sosny zwyczajnej trzech gatunków grzybów siniznowych (*Ophiostoma minus*, *O. piceae* i *Leptographium procerum*) związanych z żerdzianką sosnówką (*Monochamus galloprovincialis*).

55-letnie sosny (*Pinus sylvestris*) były inokulowane dwutygodniową grzybnią wyrosłą na 2-procentowej pożywce agarowo-maltozowej. Dla celów kontrolnych drzewa inokulowano sterylną 2-procentową pożywką agarowo-maltozową. Każde drzewo było inokulowane jednym gatunkiem grzyba, przy gęstości inokulacji 400 lub 800 punktów na 1 m^2. Na każdym drzewie inokulacje obejmowały 60-centymetrowy pas zlokalizowany na wysokości pierśnicy. Liczba punktów inokulacyjnych wynosiła od 60 do 144. Po 23 tygodniach od inokulacji drzewa były ścinane i z każdego z nich wycinano 1,2-metrowe wałki zawierające punkty inokulacyjne. W laboratorium z każdego wałka usuwano korę i mierzono długość oraz szerokość nekroz powstałych wokół punktów inokulacyjnych, z wyciętych krążków zaś obliczano powierzchnię tzw. suchych stref. Żadne zakażone grzybami drzewo nie wykazało objawów zamierania. Drzewa inokulowane grzybem *O. minus* wytworzyły największe nekrozy w drewnie bielastym. Wielkość tych nekroz odpowiadała wielkości suchych stref powstałych w drewnie bielastym. Grzyb *L. procerum* spowodował także wyraźnie dłuższe nekrozy oraz więcej suchych stref w drewnie bielastym niż w kombinacji z grzybem *O. piceae* oraz w kombinacji kontrolnej. Zarówno *O. minus,* jak i *L. procerum* generowały krótsze nekrozy przy 400 punktach inokulacyjnych na 1 m^2 niż przy 800 punktach, jednak różnice te nie były istotne statystycznie.

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Authors' addresses:
Dr. eng. Robert Jankowiak, University of Agriculture in Cracow, Department of Forest Pathology, Al. 29 Listopada 46, 31-425 Cracow, Poland, e-mail: rljankow@cyf-kr.edu.pl
Dr. eng. Robert Rossa, University of Agriculture in Cracow, Department of Forest Entomology, Al. 29 Listopada 46, 31-425 Cracow, Poland
Dr eng. Piotr Bilański, University of Agriculture in Cracow, Department of Forest Protection and Forest Climatology, Al. 29 Listopada 46, 31-425 Cracow, Poland

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