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ORIGINAL RESEARCH PAPER

Leaf-spot disease on European mistletoe (*Viscum album*) caused by *Phaeobotryosphaeria visci*: a potential candidate for biological control

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Abstract *Viscum album* (European mistletoe), a perennial, evergreen, hemiparasitic shrub, infects a wide range of woody species. It adversely affects the height and diameter of growth and it is associated with increased mortality of its hosts. There is no effective control methods against it. We have found a specific hyperparasitic fungus, which can completely destroy European mistletoe by infecting its branches, leaves and berries. Both morphological and molecular identification, based on ribosomal internal transcribed spacer sequences (rDNA-ITS), established its identity as *Phaeobotryosphaeria visci*. Our analysis also revealed unexpected ITS variability, as compared to the previous studies, that needs to be considered in identifying of this pathogen. Because of its efficient pathogenicity this fungus might be a good candidate for biological control of mistletoe.

Keywords Botryosphaeriaceae · Fungal diversity · Hemiparasitic plants · Internal transcribed spacer · Mistletoes · Phytopathogenic fungi

Introduction

European mistletoe (*Viscum album* L.) is an evergreen, perennial, epiphytic, hemiparasitic shrub, which is a plant pathogen, a source of pharmaceutical

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compounds and a symbol in mythology (Zuber 2004). It infects more than 450 woody plant species (Barney et al. 1998) and is widely distributed in Europe. It was introduced to North America and Canada at the beginning of the 20th century (Hawksworth et al. 1991). European mistletoe damages forest and ornamental trees, as well orchards. It affects negatively the height and diameter growth, and lowers the vigor of the host—inducing premature mortality. Furthermore, it adversely affects the quality and quantity of the wood, reduces fruiting, and predisposes their hosts to be attacked by other agents, such as insects or decay fungi (Hawksworth 1983). Reid et al. (1994) confirmed that the infected trees had significantly higher mortality than trees where *V. album* has been removed.

Several means of controlling mistletoes have been tested but direct methods, such as pruning of infected branches, or removing infected trees, are still the only effective methods. Systemic herbicides, such as 2,4-D, 2,4-5 T, 2,4-MCPB, and dichloroethane, kill *V. album* shoots on *Abies* with little host damage, and further tests on various deciduous trees have been promising (Delabraze Par and Lanier 1972; Hawksworth 1983). Baillon (1988) reported experiments with 2,4-DB and glyphosate but they mostly focused on the translocation and penetration of these herbicides in the host trees. They found no herbicide in the host but observed that maximum effects lasted only 4–6 months.

We started to look for an effective control method by studying fungal pathogens infecting European mistletoe. Over 20 microscopic fungi live on European mistletoe, but only a few of them cause major damage on the plant (Karadžić et al. 2004). We found and isolated one fungus which can infect the entire hemiparasitic plant, including berries, leaves and branches. After infection it induces the total necrosis of the shrub and haustoria. Our aim was to identify this fungus and to survey its potential for biological control. The morphological identification, coupled with the genetic analysis, and the pilot pathogenicity tests are reported.

Materials and methods

Sampling, culture and DNA isolation

Diseased European mistletoe leaves were collected at Keszthely, Hungary in August 2010 from three host trees (*Acer saccharinum* L., *A. pseudoplatanus* L. and *Populus nigra* L.). Samples were separately placed in a wet chamber and incubated for one day at 25°C to swell the conidiomata. Thirty conidiomata were carefully excised from each leaf sample and suspended in 800 µl sterile distilled water. From this suspension, 250 µl triplicates were spread on PDA plates (4 g potato extract, 20 g glucose, 15 g agar per liter) and incubated at 25°C for 24 h. Germinating individual spores were placed on PDA plates, containing 30 mg kanamycin and 100 mg ampicillin, and incubated for 1 week at 25°C. Final subcultures were made for DNA purification from disks 5 mm diam. excised from the proliferation zone of the colonies, which were placed on a semi-permeable plastic membrane ensuring the isolation of mycelia. DNA extraction was initiated 10 days after the last passaging and followed the CTAB method of Zhang et al. (1996).

PCR amplification and sequencing

Molecular identification of the isolates was performed using the sequence data of the Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA. The amplification was performed with the primers ITS1 and ITS4 described by White et al. (1990). Amplification reactions were performed with twenty isolates in 50 µl containing 25 µl nuclease-free water, ~20 ng template DNA, 0.5 µM each primer, 0.2 mM dNTP, 5 µl 10× PCR buffer (1 mM Tris/HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100) and 0.5 U Taq polymerase. PCR was performed using 2 min at 94°C for initial denaturation, 35 cycles of 30 s denaturation at 94°C, 1 min annealing at 50°C, and 2 min extension at 72°C, followed by a final extension for 5 min at 72°C. Amplification products were separated on 1.5% agarose gels stained with ethidium-bromide. Fragments excised from agarose gels were cleaned and sequenced directly in both directions using the primers ITS1 and ITS4.

ITS sequence alignment and parsimony analysis

The sequences of the phytopathogenic fungus were used for a nucleotide blast (blastn) search with a maximum of 100 target sequences and with the expected threshold of 10 performed at NCBI. Further

sequences showing high similarity to *Phaeobotryosphaeria visci* were also downloaded for the analyses together with multiple outgroup terminals from the clades of Botryosphaeriaceae (see Supplementary Table 1). Sequences were aligned with ClustalW within BIOEDIT (http://www.mbio.ncsu.edu/bioedit/bioedit.html) using the default parameters of a match score of 5, a mismatch penalty of 4, a gap open penalty of 15 and a gap extension penalty of 6.66.

Phylogenetic analysis of the sequences was made with parsimony as an optimality criterion using the program PAUP* version 4.0b10 (Swofford 2002). Uninformative characters were excluded and all the remaining characters were equally weighed. Alignment gaps were treated as missing data in the analysis that implemented tree bisection and reconstruction (TBR) as the branch-swapping algorithm with 1,000 heuristic random additional replicates saving and holding all trees. Search options included MulTrees, no MaxTrees limit, swapping on all trees, and parsimony options set to collapse branches if minimum length is zero ("amb-") with simple taxa addition. Bootstrapping was used to obtain support values for the resulting trees with 1,000 random additional replicates. Tree length, consistency (CI, Kluge and Farris 1969), and retention indices (RI Farris 1989) were calculated, and strict consensus tree was obtained and edited with the FigTree v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Morphological analysis and pathogenicity test

The morphological identification was carried out following Brandenburger (1985). Symptoms on infected mistletoe leaves, culture and spore characteristics were analyzed along with genetic experiments. Further pathogenicity tests (re-infections) were performed with re-isolation to comply Koch's postulates. Re-infections were carried out with two isolates (Pheo4, 13) on healthy and carborundum treated leaves on separate mistletoe shoot in four replicates each containing two infected leaves. Mistletoe shoots in closed floral water tubes were placed into an incubator, where relative humidity (RH) was adjusted to 75% using a glycerol/water azeotropic mixture at 20°C (Grover and Nicole 1940). Spring continental climatic conditions of the region—possibly needed for the infection of the pathogen—were simulated with this procedure. Mycelial disks (1 cm^2) from subcultures grown for 7 days on semi-permeable membrane were attached to each leaflet. Freshly infected leaves were moistened twice for the first 4 days then left intact in the incubator for 4 weeks. The pathogenicity dataset was assessed with two-way analysis of variance in the case of pycnidia number distribution with the first factor set as the type of treatment (carborundum-treated and intact leaves) while the second factor was assigned to isolates (Pheo4 and Pheo13) and the response variables were given as follows: total number

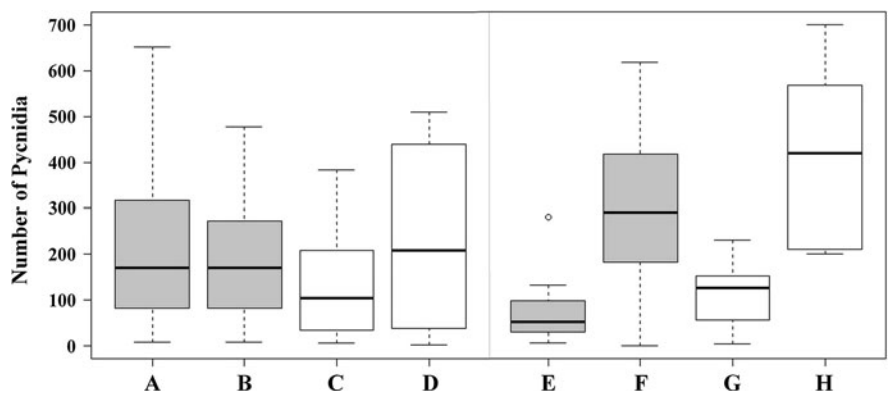


Fig. 1 Mean number of pycnida based on the analysis of variance-type statistics. *Bars* are textured according to treatment type: intact leaves (*white bars*), leaves treated with carborundum (*gray-shaded bars*). **a**–**d** isolate Pheo4, **e**–**h** Pheo13. **a**, **c**, **e**, **g** abaxial leaf surface; **b**, **d**, **f**, **h** adaxial leaf surface. *Thick line* inside the *box* indicates the median value of the data. The *lower limit* of the *box* shows the lower (25%) and the upper (75%) quartile, and therefore captures 50% of all data (inter-quartile range)

of pycnidia, number of pycnidia on the adaxial and abaxial surface of leaves. We also tested correlations among leaf area and total number of pycnida. Assumptions were tested after each analysis step.

Results

Morphological identification and pathogenicity test

Morphological identifications along with sequence data revealed that the fungus isolated from the fallen twigs of European mistletoe was *P. visci* [Syn.: *Botryosphaeria visci* (Kalchbr.) Arx & E. Müll.; anamorph: *Sphaeropsis visci* (Fr.) Sacc.] a dark-spored ascomycete of the Botryosphaeriaceae. Pycnidia found on infected mistletoe tissues were 200–300 μm and were initially light brown then turning to dark brown and black with age. Pycnidia formed on the surface of pure cultures were bigger, up to 300–350 μm in all cases, and were initially light grey turning later into dark grey or black. Conidiomata were unilocular or rarely multilocular (up to 3) where the inner layer was formed by textura angularis. Proliferating pycnoconidia were borne on yellowish-brown conidiogenous cells between hyaline paraphyses. Pycnoconidia were thick-walled, proliferating from conidiogenous cells with truncate or obtuse base, and the texture of their surface were internally finely verruculose. The pathogen developed well on PDA medium preferring a lower temperature (20°C) for mycelia development, and reaching 80–90 mm diam. after 14 days. Pycnidia appeared after 3–4 weeks or occasionally later.

In the pathogenicity test, diseased leaves showed chlorotic symptoms seven days after the infection and later became slightly white. Pycnidia started to appear 14 days after the infection in the inoculation mark and their number gradually increased together with the area of chlorotic tissues. We found a significant difference ($F = 4.99$, $p = 0.03$) among pycnidia number on adaxial leaf surface and type of the isolates. The mean number of pycnidia was 213 ± 176 ($n = 16$) in the case of isolate Pheo4, while this was 359 ± 190 ($n = 16$) for Pheo13 (Fig. 1). We found no significant differences

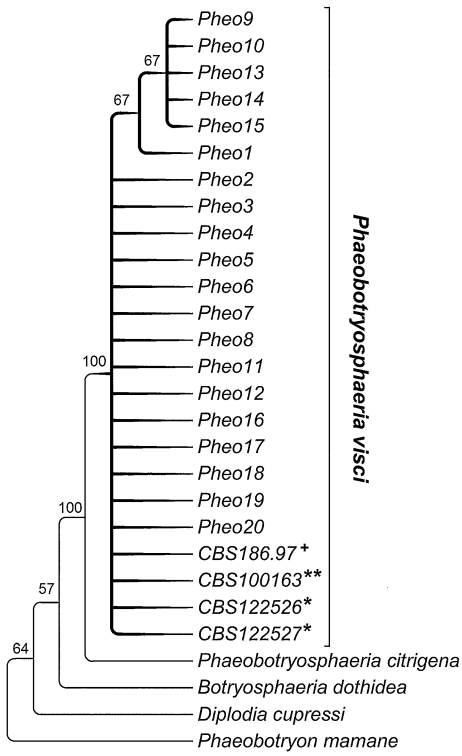
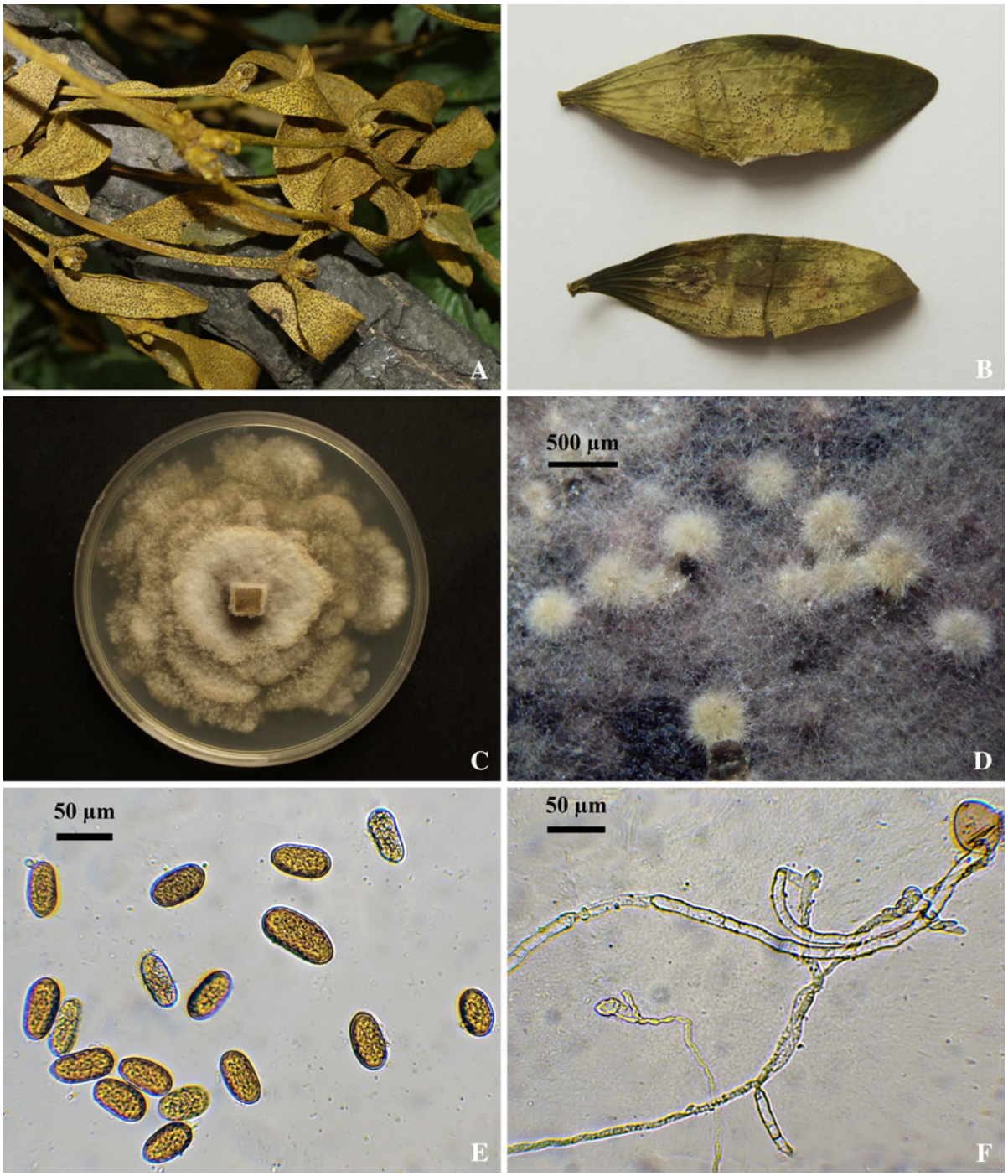


Fig. 2 Strict consensus tree of 41,444 equally parsimonious trees (EPTs) found in parsimony analysis of internal transcribed (ITS) data (consistency index = 0.925, retention index = 0.778). The *high values of the both indices* indicate good fit of the characters with the resulting trees, and relatively low level of homoplasy. All isolates were resolved within a well supported clade with four terminals of *Phaeobotryosphaeria visci* representing isolates previously deposited at GenBank by Phillips et al. (2008). Numbers above branches are bootstrap support values. *Asterisk* indicate the locality of *P. visci* isolates: +Germany; *Ukraine; **Luxemburg

Fig. 3 **a** Infected European mistletoe (*Viscum album* L.) leaves on *Populus nigra* L. showing yellow chlorosis with immersed to erumpent and superficial (beneath the epithelial layer of host tissues) pycnidia forming on stems, leaves as well as on the berries reaching densities of 25–70 per cm^2. **b** Pathogenicity test: symptoms of *Phaeobotryosphaeria visci* on mistletoe 2 weeks after inoculation. **c**: *Phaeobotryosphaeria visci* culture grown for 14 days. Surface pigmentation was initially white then becoming dirty grey while the underside was black with radially growing, zonated or lobed (rosette-like) cultures with undulated margins. **d**: Mature pycnidia on the surface of pure cultures. *Scale bar* 500 μm. **e** Half-mature conidia of the pathogenic fungus. Aseptate conidia were moderately thick-walled, 45–55 × 20–24 μm, greenish yellow in and later becoming dark brown, sometimes scattered with fine oil droplets with an obtuse apex (Photo: Cs. Pintér). **f** 24-h old germinating spore with a 250–500 μm germ tube. The germination of pycnoconidia usually started after 8–12 h at 25°C (Photo: Cs. Pintér) ▶



($p > 0.05$) in other cases. Details are given in Supplementary Table 2. After correlation analysis, we concluded that there was no linear or either monotone correlation among leaf surface area and mean number of pycnidia, indicated by low Spearman's rank and Pearson's correlation coefficients ($r = 0.26$ in both cases), fulfilling assumptions and making results undoubtedly acceptable.

Sequencing and parsimony analysis

PCR amplifications yielded one clear band with no length variation indicating lack of alternative ITS copies from paralogous loci. The length of the ITS sequences of isolates ranged from 560 to 562 bp, containing additional T (isolates Pheo9, 10 and 14) or GT (Pheo13) bases at the aligned positions 70–71. The complete alignment is provided as Supplementary Fig. 1. Length variations were also due to additional C at 81 position (Pheo1) or (A)CT(G) bases at the aligned position 88 (Pheo4)(see Supplementary Table 3). Sequencing in reverse directions with primer ITS4 confirmed the presence of these indels. The final alignment contained 28 terminals and 621 characters, 552 characters were constant while 53 variable, but only 16 were parsimony-informative. The strict consensus trees calculated from these is shown in Fig. 2.

Discussion

Our analysis revealed unexpected ITS variability in *P. visci* as compared to previous studies (Phillips et al. 2008). Our results can be used in diagnostics to identify this pathogen. All insertions/deletions, or further single nucleotide polymorphisms (SNPs), were found in the sequence of ITS1. We have found no mutations in the intercalated 5.8S ribosomal exon, or in the sequence of ITS2, indicating further presence of putative compensatory base changes. This can be explained by the results that only the ITS1 region accumulates numerous SNPs in its sequence, as compared to the ITS2 or to the 5.8S rDNA gene. Previous studies revealed no polymorphism among isolates of *P. visci*. Four sequences were obtained from different isolates from GenBank, and they all fall into one group with the majority of sequences produced in this study. Two of the deposited isolates originated from Ukraine while the two other were from Germany and Luxemburg, respectively. Despite of the geographic distance between host plants, the gained sequences were identical with each other. This, coupled with our data, could indicate that genetic diversity might be low within populations. In further studies sampling should be extended within each region involving more isolates from each location in order to capture rare haplotypes (or alleles) present in populations with lower frequency. This sampling strategy would possibly help to overcome population bias produced by frequent haplotypes (or alleles) in further population genetic studies. Molecular identification of *P. visci* using the ITS region appears to be reliable as the sequences were similar, but still showed parsimony informative sites distinguishing different haplotypes. This region can be used in further studies aiming to reveal nucleotide diversity and population structure of this fungus at wider scale. Our results are an initial attempt to assess the overall diversity of the pathogen on larger geographical scale in order to begin

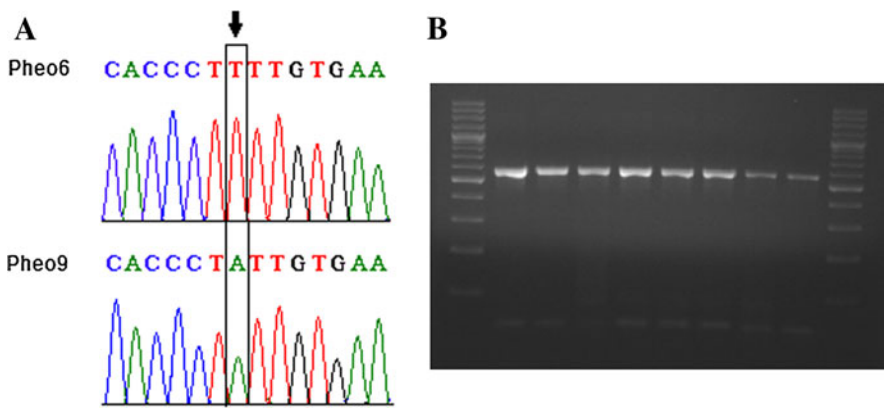


Fig. 4 **a** Partial electropherograms obtained for Pheo6 and Pheo9 isolates showing an exemplar T/A transversion mutation at the aligned position 70 (see alignment at Supplementary Table 3). Sequencing reads returned clear, unambiguous electropherograms in both directions, indicating that concerted evolution left no trace of inappropriate homogenization leading to the selection of alternative copies. **b** PCR amplification patterns of the ribosomal internal transcribed spacer (rDNA-ITS) region among isolates (from left–right, Pheo1–8), showing one clear 560 bp size fragment cleaned and used for sequencing; indicating that the identification of isolates was based on dominant orthologous ribotypes

to understand the population biology of this disease. The fact that we were able to detect polymorphism within one population, as compared to previous studies, could indicate that a denser sampling and analysis in regions where this pathogen is widely distributed could reveal more information about the diversity and reproduction of this fungus (Figs. 3, 4).

In summary, both morphological and molecular identification, verified by Koch's postulates, proved that the disease capable to completely destroy European mistletoe is caused by the phytopathogenic fungus *P. visci*. For successful infections injuries are not necessarily needed on the leaf surface contradicting the findings of Karadžić et al. (2004) who achieved successful inoculation only with mechanically damaged leaves. In addition to their findings we observed disease symptoms also on intact leaves indicating that abiotic factors (i.e. temperature, RH%) are key issues of successful infection. However, further histopathological analysis is needed to understand the complete mechanism of infection. With this agent we might be able to develop the first effective tool of protection against mistletoe, and use it in biological control. This management strategy against *V. album* would have several advantages, since biological control is not just environmental friendly, but it can be applied in areas, where pesticide spilling is impossible, e.g., urban areas, or next to kindergartens. To achieve this goal we need to understand the biology of the host- hemiparasite- hyperparasite (tree–mistletoe–fungi) relationship. At present, practically nothing is known about the biology, genetics, reproduction, interaction and coevolution of *P. visci* and *V. album*. We are working on these aspects in order to gain understanding of the complexity of this pathogenic system.

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