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IN SITU INOCULATION OF LARCH WITH THE THREATENED WOOD-DECAY FUNGUS *FOMITOPSIS OFFICINALIS* (BASIDIOMYCOTA) – EXPERIMENTAL STUDIES

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Abstract. Pilot studies aimed at devising methods of active *in situ* protection of *Fomitopsis officinalis* (Vill.: Fr.) Bond. & Sing. are described. Thirty healthy larches in the Rudka Sanatoryjna Nature Reserve and 20 larch stem sections in an open area of the Mińsk Forest District were inoculated with larch wood overgrown with *F. officinalis* mycelium. This trial of artificial infection can be considered successful, as live mycelium of *F. officinalis* was recorded from cut test trees and 14 stem sections three years after the experiment began.

Key words: active protection, tree inoculation, *in vitro* culture, PCR-RFLP

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INTRODUCTION

Fungi constitute one of the most numerous groups of living organisms in terms of species, but many are acutely threatened. The threat is attributable to global environmental changes caused by extensive human activity, the cutting of individual old trees and entire stands, air, water and soil pollution, disturbance of the water cycle, and trampling and littering in forests. As a result, some fungal species have already become extinct and others may soon disappear.

Legal protection and urgent mycological studies on endangered and vulnerable species should develop together with habitat protection of fungi to remedy this situation. The establishment of national and regional networks of protected areas, aimed at preserving biological diversity, encourages conservation of fungi (Grzywacz 1999). Some fungal species are known to be associated exclusively with long-lived trees, found in such protected areas. Active protection of selected species of protected and threatened fungi can also be implemented. Poland's regulation on protected species of wild-growing fungi of 9 July 2004 allows the reintroduction of fungi cultured *ex situ* to the natural environment, but not enough is known about the methodology and results of transferring fungi to new places.

Fomitopsis officinalis (Vill.: Fr.) Bond. & Sing. is undoubtedly a species acutely threatened with extinction in Poland. Its supposed medicinal properties, for which the fungus has been sought and collected, have contributed to its disappearance. The fungus was believed to cure all diseases, including cancer (Rządkowski & Sabiniewicz 1936; Muszyński 1954; Knopf 1984; Semerdžieva & Veselský 1986). Also, the demand for larch wood and non-wood plant substances (resin, tanbark) has led to excessive exploitation of larch forests (Kluk 1808, Barański 1963) and their disappearance.

Carpophores of *Fomitopsis officinalis* are perennial, living as long as fifty years or more; they are sessile, usually ungulate or cylindrical after many years of growth, sometimes growing together and forming irregular concentrations (Domański *et al.* 1967). They usually appear a few decades after attack (Konev 1972) at the site of the initial infection of the oldest (most developed) rot center. Infection usually takes place through knobs or through exposed heartwood where mechanical damage has occurred. The spores germinate into

mycelium which reaches the wood interior and develops further. *F. officinalis* causes intense brown rot, mostly of heartwood, which first cracks along the rays and annual rings, and later crumbles into tiny cubes. The cracks are filled with whitish, patchy mycelium (Zhuravlev *et al.* 1974; Fedorov 1987).

The species is distributed in the entire temperate zone of the Northern Hemisphere. It was reported from Africa (Morocco), North America (Canada, U.S.A.), Asia (China, India, Japan, Korea, Mongolia, Russia) and most European countries (Ryvarden & Gilbertson 1993; Chlebicki 2001; Chlebicki & Łuszczyński 2002; Chlebicki *et al.* 2003; Dahlberg & Croneborg 2003).

It is frequently noted on trees of the genus *Larix* and less often on *Pseudotsuga, Abies, Pinus, Picea, Tsuga* and *Cedrus*. In Europe, outside Spain, where it was found on *Pinus nigra* subsp. *salzmannii*, it occurs exclusively on larches: *Larix decidua*, *L. decidua* var. *polonica* and *L. sibirica* (Domański *et al.* 1967; Browne 1968; Kotlaba 1984; Ryvarden & Gilbertson 1993; Łuszczyński 2000).

Fomitopsis officinalis is now very rare all over Europe. According to Dahlberg and Croneborg (2003), the greatest number of localities after 1980 has been reported from France and Switzerland (40 and 30 localities, respectively), and a few populations have been confirmed in Austria (4), Germany (4), Slovenia (3), Poland (2) and Romania (1). Inspections of 43 forest stands conducted in Poland between 1998 and 2003, including 41 nature reserves with larches, showed the occurrence of *F. officinalis* at 5 localities and on 17 trees (Piętka & Szczepkowski 2004). Because of the rarity of its occurrence in Poland, *F. officinalis* has been protected since 1983 (Grzywacz 1989). It is also red-listed as an endangered species (E) whose survival is very unlikely if threat factors persist (Wojewoda & Ławrynowicz 1986, 1992). The Council of Europe recommends it for inclusion on the European list of protected fungi (Anonymous 2001).

Active *ex situ* and *in situ* protection of acutely threatened fungi has become an increasingly essential element of biodiversity conservation methods. The aim of the present project was to assess the feasibility of infection of living larches and dead wood with *F. officinalis* mycelium in natural conditions, as the first step towards devising effective strategies of *in situ* protection of *F. officinalis.*

Material and methods

IN VITRO CULTURE OF *F. OFFICINALIS*

One carpophore of *F. officinalis* was acquired from the Chełmowa Góra Protected Area in the Świętokrzyski National Park. The carpophore (Fig. 1) grew on a larch over 100 years old, at the height of 2.5 m on the north side of the trunk. The fungus was cut out with a sizeable wood fragment and transported to the laboratory of the Department of Mycology and Forest Phytopathology, Warsaw Agricultural University. Pure culture of the *F. officinalis* mycelium was obtained from fragments of the infected wood directly beneath the carpophore. The mycelium was cultured in Petri dishes (7 cm) on agar-wort medium with larch sawdust (50 g sawdust per liter medium). Every 2–3 months the mycelium was inoculated onto new medium. The dishes were incubated for *ca* 4 weeks in a Heraeus BK 600 incubator at 22°C and then refrigerated.

Mycelium-overgrown chips of larch heartwood (30 × 5 × 5 mm) prepared in the laboratory were further used *in situ* as inocula. The wood pieces were prepared as follows: 30 ml distilled water with brewer's wort (3:1) was added to 200 ml flasks containing *ca* 50–60 wood pieces each (up to 1/3 the height of the flask) and then the flasks were autoclaved at 121°C for 30 min. The wood pieces were inoculated with *F. officinalis* mycelium one hour after sterilization (a fragment of mycelium-overgrown medium from the cultures in Petri dishes was placed in each flask), and the flasks were incubated at 22°C for *ca* 2 months.

ARTIFICIAL INFECTION OF STEM SECTIONS AND TREE TRUNKS IN NATURAL STANDS

The field experiment was established in September 1999 and consisted of two parts: inoculation of dry larch stem sections, and inoculation of living larch trees. Twenty 60 cm long sections of larch stem were prepared and dried. They were placed vertically in two rows under the canopy of a fruit tree. The sites were set up in the village of Maliszew near the town of Mińsk Mazowiecki.

In the second part of the experiment, thirty living larches were inoculated in the Rudka Sanatoryjna Nature Reserve located in the Mińsk Forest District (area

center: 52°09'46"N, 21°48'58"E). According to a forest management planning report (1996–2005) the larches were over 110 years old. The trees selected for inoculation were located in forest tracts 80 and 82. The trees were marked permanently with galvanized iron plates fixed to the trees at 2.5 m the height on the side facing the road. The mean diameter at breast height (dbh) of the inoculated larches was 44.9 cm.

Inlet holes were drilled in both stem sections and trees; they were drilled 8 mm in diameter and 10–15 cm deep in order to reach the heartwood. One inoculum was introduced with tweezers into each inlet hole. The holes were closed up with a freshly cut, debarked shoot of *Corylus avellana*, *ca* 30 mm long.

The stem sections were inoculated halfway up their height from the ground, at the N, S, E and W points. The trees were inoculated in two series at heights of 2 and 3 m, at the N, S, E and W points. Inoculations were performed on a sunny day.

The experiment involving the stem sections was terminated after 3 years. The logs were split along lines delineated by the drill holes. Pieces of wood properly described and packaged were transported to the laboratory. In October 2002, two of the 30 larches inoculated in the Rudka Sanatoryjna Reserve were also cut for the first test. Two one-meter trunk sections (at 1.5–2.5 m and 2.5–3.5 m) were taken from both trees and transported to the laboratory, where they were split. The original inoculum was taken out in the inoculation chamber, and wood samples were collected every 2 cm along the trunk, starting at 1 cm from the inoculation point, that is, at 1 cm, 3 cm, 5 cm, etc. (15 sampling sites in one direction from the inoculation point). The material collected was placed on Petri dishes with agar-wort medium to test for the presence of fungi. The obtained cultures were compared morphologically with the original mycelium of *F. officinalis*. Selected cultures (one culture from each cut tree) were additionally compared by PCR-RFLP technique.

PCR-RFLP ANALYSES OF MYCELIUM

The mycelium was identified by comparison of the PCR-RFLP profiles of the ITS region of rDNA. The test material was derived from *ca* 1 cm^2 mycelium from the respective *in vitro* cultures, placed in CTAB buffer (300 µl) for subsequent DNA extraction. Three groups of mycelial cultures were taken for comparative analysis: mycelium extracted from the tree at the base of the carpophore of *Fomitopsis officinalis* originally taken from the Chełmowa Góra Protected Area (and later used for experimental inoculation of trees), mycelium developed in the first inoculated tree examined,

Fig. 1. Fruitbody of *Fomitopsis officinalis* (Vill.: Fr.) Bond. & Sing. with the wood fragment acquired for the experiment.

and mycelium developed in the second inoculated tree examined. Additionally, a small fragment of the hymenium from a dried herbarium specimen (WAML) of *F. officinalis* was taken for a reference DNA profile (one sample of each type was taken).

The general analysis procedure followed the methodology described, for example, by Ronikier *et al*. (2002). DNA was isolated from the mycelium using the modified CTAB method, including mechanical grinding of tissue in the CTAB buffer, incubation at 65°C (cell membrane lysis), mixing with cold chloroform and extracting DNA with the upper phase, DNA precipitation with isopropanol, washing the pellet with sodium acetate-ethanol solution followed by 80% ethanol, and resuspension in 50 µl deionized water.

Amplification was performed using ITS1/ITS4 universal primers for the ITS region of ribosomal nuclear DNA (Gardes *et al.* 1991). The reaction mixture (final volume 20 µl) contained PCR buffer (10x, Qiagen) – 2 µl, $MgCl_2$ (25 mM) – 1.5 mM, dNTP (2 mM, Sigma) – 1.25 mM (of each: dATP, dCTP, dGTP, dTTP), primers (10 µM) – 0.5 µM (of each: ITS1 and ITS4), Taq polymerase (5 U/µl, Qiagen) – 0.4 U, DNA extract

– 2 μl, and ddH_2O – up to 20 μl. The PCR profile was as follows: 5' at 94°C; (30" at 94°C, 1' at 52°C, 1.5' at 72°C) 32x; 5' at 72°C; storage at 4°C.

For restriction analysis, enzymes HapII, HaeIII and EcoRI (Amersham Biosciences) were used. Amplified DNA was incubated overnight with single enzymes; the reaction mixture included the 10x enzyme-specific buffer – 1/10 of the final volume, restriction enzyme (10U/μl or 15 U/μl) – 3 U, BSA (10 ng/μl) – 0.6 μl, amplified DNA solution – 3 μl, ddH_2O – up to 17 ddH_2O. Restriction products were separated by electrophoresis on 2.0% agarose gel. Restriction profiles were compared using GeneTools software (Syngene); the molecular weight of bands was assigned by comparison with the GeneRuler 100bp DNA Ladder (Fermentas).

Results

Development of *F. officinalis* mycelium in stem sections and trees

Laboratory dish tests showed the presence of *F. officinalis* mycelium in the wood of 14 stem sections. One section had symptoms of the most advanced brown rot, with wood visibly crumbling into prismatic blocks up to 18 cm from the inoculation point in one direction parallel to the trunk axis, and 7 cm radially (total measurement). Mycelium was isolated at depths of 5, 7, 9, 11, 13, and 17 cm from this log. Visible rot was recorded 5–9 cm from the inoculation point along the log axis in the remaining 13 colonized sections. No mycelium was isolated from six sections.

Fruitbodies of *Bjerkandera adusta* were additionally recorded on 11 of the 20 inoculated larch stem sections upon termination of the experiment, but they occurred exclusively within sapwood.

Two living larches were acquired in the Rudka Sanatoryjna Reserve. The trees to be cut were designated by the local forester, respecting conservation imperatives. Laboratory dish tests showed that it was possible to re-isolate *F. officinalis* mycelium from the first tree (dbh 41 cm) at the level 3 m from one drill hole, from a section at 1 cm from the inoculation point, and from the inoculum, but attempts to re-isolate mycelium from the other inlets failed. One of the inlet canals and the inoculum on the sapwood side at the level of 2 m were completely covered with resin. In the case of the second tree (dbh 36 cm) at the level of 2 m, re-isolation from the inoculum and from sections at 3, 7, 9, 13 and 15 cm from the inoculation point was successfully performed. Visible wood rot reached 17–18 cm from the inoculation point in one direction parallel to the trunk axis. Mycelium also survived in the inoculum collected from another inlet hole. In the 3 m height series, the presence of *F. officinalis* mycelium was recorded in two cases. In the first case, mycelium from the inoculum and from the wood was successfully re-isolated from 1, 3, 5, and 9 cm from the inoculation point (wood rot visible up to 10–11 cm from that point). In the second case, mycelium was isolated from the inoculum, and was additionally isolated from the wood at 1, 3, and 5 cm from the inlet hole; wood rot symptoms were visible 8–9 cm from the inoculation point.

Identification of mycelium by PCR-RLFP analysis of rDNA ITS region

The ITS region was successfully amplified in all DNA isolates from the cultures. It was not possible to obtain DNA extract directly from the herbarium specimen. A mycelium-based culture from the base of the carpophore therefore served as a first source of *F. officinalis* DNA. The three enzymes used generated 3- or 4-band restriction profiles, and proved the identity of the mycelium from the base of *F. officinalis* carpophore with the mycelium that developed in the two artificially inoculated trees (Table 1).

Discussion

Fomitopsis officinalis causes brown rot of heartwood in natural conditions (Ryvarden & Gilbertson 1993). Heartwood in the trunks of *Larix polonica* growing in the Góra Chełmowa Protected Area has been found to occupy 80–90% of the surface of a transverse section (Krzysik & Gonet 1961). Such an abundance of nutritive substrate allows *F. officinalis* mycelium to develop inside the trunk for decades without producing carpophores.

Sometimes rot can also be observed in sapwood (Zhuravlev *et al.* 1974; Semenkova & Sokolova

Table 1. Results of PCR-RFLP analysis of rDNA ITS region in *Fomitopsis officinalis* (Vill.: Fr.) Bond. & Sing.

	Fragments after restriction with enzymes (length in bp)		
	HapII	HaeIII	EcoRI
Mycelium from the base of carpophore (*in vitro* culture)	422.95 184.95 80.90	532.20 118.30 72.95	311.30 273.50 150.60
Mycelium developed in the first inoculated tree (*in vitro* culture)	422.95 184.95 80.90	531.35 118.30 71.45	311.30 278.60 150.70
Mycelium developed in the second inoculated tree (*in vitro* culture)	422.95 186.20 81.10	531.35 118.30 72.95	311.10 278.70 151.00

1992). Laboratory studies have shown that physiologically inactive sapwood with proper moisture (*ca* 66%) is a more favorable substrate than heartwood for the development of *F. officinalis* (Piętka 2004). Compared with heartwood, sapwood is less saturated with resin, its specific gravity is less, and the parenchyma cells contain a number of organic substances that are a source of nutrition for the mycelium (Krzysik 1974); consequently, it is decomposed faster.

The high effectiveness of *F. officinalis* inoculation of the larch stem sections may be attributed to low competition from saprotrophic fungi in the experimental environment. Only fruitbodies of *B. adusta* were noticed on the dried stem sections, and only within sapwood, while *F. officinalis* could develop freely in heartwood. Re-isolation of mycelium from the stem section with the most intense rot showed that *F. officinalis* had reached up to 18 cm from the inoculation point in one direction parallel to the trunk axis during the three years of the experiment. Borzini (1941) reported that *F. officinalis* can produce carpophores on lying tree trunks and larch stumps. According to Boyce (1961), it may occasionally cause wood decomposition in timber yards, although only when building timber is made from already infected wood. Unfortunately, *F. officinalis* carpophores were not obtained in the experiment, possibly because of its short duration.

This trial artificial infection of living larches in the Rudka Santoryjna Reserve should be considered successful, as *F. officinalis* mycelium was recorded in both cut test trees after the three years of the experiment. The identity of the mycelium was confirmed in by comparative DNA analysis (PCR-RFLP technique) of *F. officinalis* mycelium re-isolated from trees inoculated with mycelium initially isolated from a tree in the Chełmowa Góra Protected Area.

Re-isolation from one drill hole in the first tree was successful, and also from three drill holes in the second one. The success of artificial inoculation was initially uncertain, as a strong flow of resin from the sapwood of the drilled trees was observed on the day the experiment was established. However, the inoculum was introduced further into the heartwood, which did not have physiological functions. Krzysik (1974) reported that resin galls die during the formation of heartwood in the wood of conifers, and that the resin occurring in them is transformed into a solid that impregnates cell membranes and fills up free spaces. When the stem sections were split, it was observed that excessively resin-saturated heartwood fragments presented a difficult obstacle for the mycelium, which died having decomposed the inoculum. In one case, the inoculum was covered by liquid resin oozing in from the sapwood side. However, the quick resin reaction (with resin flowing outside) was beneficial, as the inlet hole was sealed up, and the risk of possible infection by other organisms was limited.

Successful re-isolation of mycelium from the place with the most intense rot in the second tree shows that *F. officinalis* had colonized the wood

17–18 cm in one direction parallel to the trunk axis during the 3 years of the experiment. Thus, *F. officinalis* mycelium may develop in duramen at the rate of *ca* 6 cm per year in the direction parallel to the trunk axis, although it usually grows significantly more slowly. As shown, the rates of its penetration into the heartwood of the growing trees and the logs were similar. Mańka (1998) reports that *Phellinus pini*, encountered fairly often in Polish forests, grows along the tree stem in heartwood (of pines) at the rate of 10–18 cm per year on average. In comparison, *F. officinalis* colonizes the substrate slowly.

Although only 2 living trees and 20 dried stem sections were analyzed, the results are promising and should encourage active *in situ* protection of *F. officinalis* as well as other lignicolous fungi.

Conclusions

1. A pure culture of *Fomitopsis officinalis* mycelium was obtained. The culture, stored in the laboratory of the Department of Mycology and Forest Phytopathology, Warsaw Agricultural University, can serve as a regional gene bank of the species.

2. Artificial infection of larches in the Rudka Sanatoryjna Reserve was successful: living *F. officinalis* mycelium was recorded in both cut test trees after the three years of the experiment, and carpophores of this rare fungus will likely be produced one or more decades later.

3. *Fomitopsis officinalis* can develop in artificially inoculated dried larch stem sections.

4. *Fomitopsis officinalis* mycelium can develop in heartwood of living trees and logs at the rate of *ca* 6 cm per year in the direction parallel to the trunk axis, though it usually develops significantly more slowly.

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