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La France disease of the cultivated mushroom *Agaricus bisporus* in Poland

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Summary. – Presence of the virus associated with La France disease was confirmed in the mushrooms collected from different farms located in Western Poland. Double-stranded RNA (dsRNA) was isolated from the mushrooms exhibiting a wide range of the disease symptoms including premature veil opening, brown-colored mushrooms, and loss of crop yield. The presence of dsRNA molecules (M1, M2, and L3) was confirmed by RT-PCR and sequencing. Furthermore, La France isometric virus (LFIV)-like particles were observed in the mushrooms extracts in electron microscopy. The LFIV infection was found in 120 of 200 mushroom samples tested. The amount of the infected samples indicated the high occurrence of La France disease that could be a threat to the mushroom industry in Poland.

Keywords: Agaricus bisporus; dsRNA; La France disease

La France disease also known as a brown disease is one of the most serious diseases of common cultivated mushrooms. Infection of the mushrooms with LFVI causes a decay of mycelium in casing and malformation of the fruit body. The malformed sporophores have smaller caps (Siden and Hauser, 1950; Hollings, 1962; Romaine and Schlagnhaufer, 1989). The stems are elongated and sometimes slightly bent or tapered downward (Schisler *et al.*, 1967; Zaayen, 1972). The LFIV belongs to the unassigned viruses (Fauquet *et al.,* 2005). The particles of LFIV are isometric, 30 nm in diameter, and contain dsRNA species of different sizes.

In general, LFIV infection of the crop at a spawning stage can lead to a higher incidence of the disease and greater crop loss, than infection at the later stages. The severe infection significantly reduces the harvest and can lead to a complete crop failure. In addition to the LFIV dsRNA, a presence of the positive sense single-stranded RNA genome of Mushroom bacilliform virus (MBV, the family *Barnaviridae*) is associated with some outbreaks of the La France disease (Tavantzis *et al.*, 1983, Fauquet *et al.*, 2005).

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Abbreviations: LFIV = La France isometric virus; MBV = Mushroom bacilliform virus; MVX = Mushroom virus X

Agaricus bisporus is a very popular mushroom worldwide, especially in Europe and North America (Zaayen *et al.*, 1968). It is cultivated in more than 70 countries, though Poland and the Netherlands are the biggest producers of these mushrooms in Europe. LFIV infection caused extensive crop losses to the mushroom industry in Great Britain during the 1960–70s. Later on, the infecting virus was effectively suppressed and controlled by the adopting of viral hygiene practices that minimized on-the-farm production and spread of basidiospores (Grogan *et al.*, 2003). The goal of our work was to identify viruses infecting the cultivated mushroom *A. bisporus* in Poland. Here we describe the results of the identification and diagnostics of LFIV.

In Poland, the presence of La France disease was first confirmed in a farm located in the Wielkopolska region in 2003 (Maszkiewicz and Malinowski, 2003). In 2009, 200 mushroom samples were collected at the commercial production farms located in Western Poland. The mushroom samples were collected from the crops that were considered as afflicted with the disease by the growers. The samples were scored as LFIV-positive or LFIV-negative on the basis of RT-PCR reaction. In addition, the samples were examined by the electron microscopy for the presence of viral particles. Total cellular RNA was isolated from the stems of infected mushrooms using method described by Revill and Wright

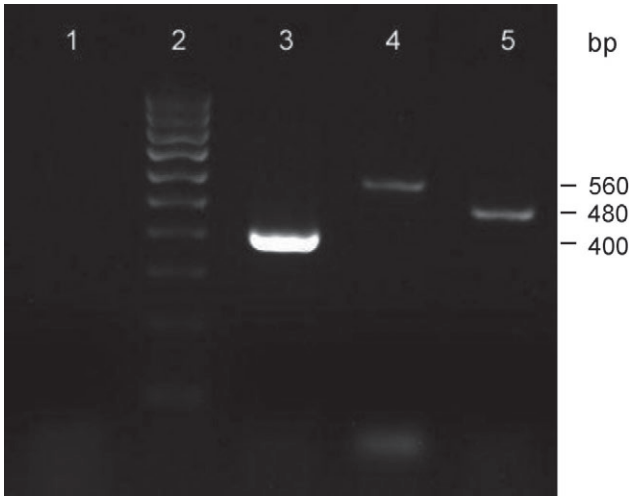

Fig. 1

Electrophoresis of RT-PCR products for M2 (lane 3), L3 (lane 4), and M1 (lane 5) using purified dsRNA as a template

Negative control (lane 1), DNA marker-Hyper Ladder IV (lane 2, Bioline).

(1997). The isolated RNA was subsequently used in RT-PCR reaction with primers designed by Revill and Wright (1997). These primers allowed to detect three independent dsRNA associated with La France disease (Harmsen *et al*., 1991). The nine disease-related dsRNAs consisting of the molecules L1 (3.6 kbp), L2 (3.0 kbp), L3 (2.8 kbp), L4 (2.7 kbp), L5 (2.5 kbp), M1 (1.6 kbp), M2 (1.35 kbp), S1 (0.86 kbp), and S2 (0.78 kbp) were characterized (van der Lende *et al*., 1994) and three of these molecules (L3, M1 and M2) were sequenced (Harmsen *et al*., 1991). Each dsRNA (M1, M2 and L3) is encoded by one large ORF. The size of the predicted products calculated from the sequence for each dsRNA molecule was 400, 480, and 560 bp, respectively. In addition, primers specific for MBV were used to check the presence of MBV RNA (Romaine and Schlagnhaufer, 1989). Total cellular RNA was denatured by heating at 95°C for 3 mins. Reverse transcription (RT) was performed with appropriate reverse primer and M-MuLV RT (Fermentas) according to the manufacturer's instructions. The PCR mixture contained 2 µl of template cDNA, 1 µl of each amplification primer (10 µmol/l stock), 10 mmol/l each dNTP, 1U *Taq* DNA polymerase (Fermentas), 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3 in a total volume of 50 µl. The reaction conditions were as follows: 94°C/3 mins, 35 cycles of 94°C/1 min, 50°C/1 min, and 72°C/1 min, and 72°C/10 mins. PCR products were separated by electrophoresis on 1% agarose gel containing ethidium bromide and visualized under UV light. The amplicons showed estimated sizes of 400, 480, and 560 bp that corresponded with the predicted sizes of PCR products. No DNA product was observed for amplifications with total cellular RNA from healthy tissues (Fig. 1). From 200 mushroom samples suspicious of LFIV infection, the positive results were detected in 120 samples. All samples were infected solely by the LFIV and accordingly, we did not detect the presence of MBV RNA.

Each of the three PCR products obtained from 20 LFIV-positive samples were cloned and sequenced. The amplified DNA was purified using Qiaex II Gel Extraction Kit (Qiagen) and subjected to the cloning into pGEM-T easy vector (Promega). Overlapping sequences were obtained using universal M13F and M13R primers. DNA for sequencing was prepared using Beckman kits as recommended by the manufacturer and sequenced using CEQ Beckman DNA sequencer. BioEdit software was used to assemble nucleotide consensus sequence. The sequences obtained from different samples were identical and therefore, sequences from one representative sample were deposited in the GenBank under Acc. Nos. GQ865514-GQ865516. The comparison of the obtained amino acid sequences with other sequences from the GenBank revealed the identity 99% for M1, 100% for M2, and 97–100% for L3, respectively. Moreover, we examined the presence of viral particles in the mushroom extracts of samples positive in the RT-PCR under the electron microscope. A huge quantity of viral particles ca. 30 nm in diameter was observed (Fig. 2).

RT-PCR analysis of the mushroom samples collected from commercial mushroom farm (Western Poland) showed that LFIV was predominantly associated with the La France disease. In this study, all 200 mushroom samples were provided by growers, who suspected the involvement of the La France disease in their crops. More than 100 samples were infected with LFIV, what was confirmed by the demonstration of the LFIV dsRNA in mushroom extracts. Currently, we do not know whether the remaining LFIV-negative samples were symptomatic due to the viral infection, environmental factors or genetic disorders of *A. bisporus*. In recent years, a novel viral disease has become prevalent in mushroom industry caused most likely by the Mushroom virus X (MVX, unassigned virus) (Fauquet *et al*., 2005). However, the characterization of its dsRNAs was lacking (Gaze *et al*., 2000). The symptoms of MVX disease were similar to the La France disease including a premature veil opening and crop delay. Mushrooms afflicted with MVX disease harbored a variety of dsRNA elements ranging between 0.64 to 20.2 kbp. The number and size of dsRNAs and the variation in their profiles suggested that MVX might comprise a complex of two or more viruses (Grogan *et al*., 2003). Nevertheless, the occurrence of additional dsRNA elements in mushroom samples collected in Poland has been observed. however, further experiments are required to confirm the association of LFIV infection with MVX.

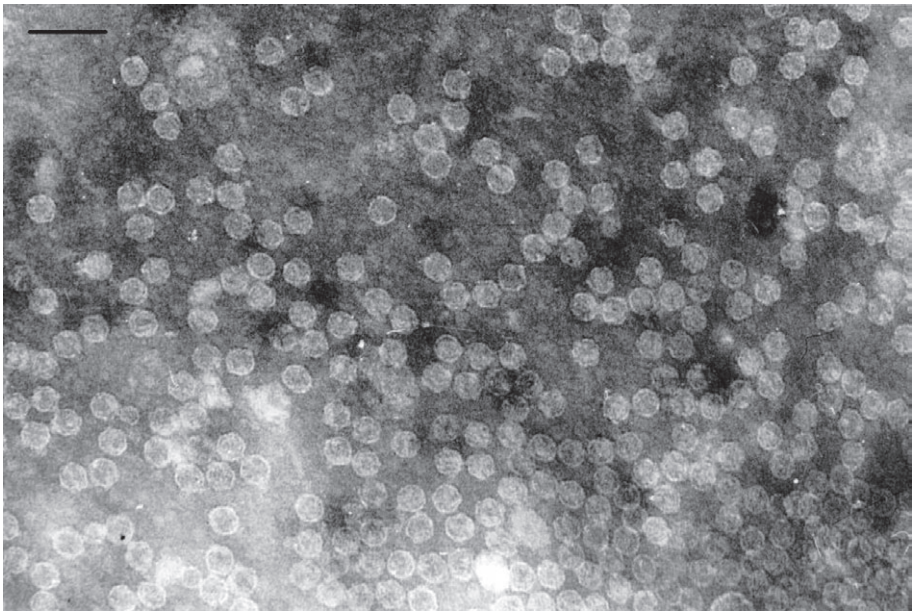

Fig. 2

Electron microscopy of LFIV particles

The bar represents 90 nm.

Our results showed the increasing threat of LFIV infection for the mushroom industry in Poland. The occurrence of la France disease in other European countries is now extremely low due to the adopting of viral hygiene practice that minimizes on-the-farm production and spread of basidiospores (Grogan *et al.*, 2003). As shown in this work, the appearance of La France disease in Poland represents a high economical risk that should be considered in the efficient control strategies against this disease.

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