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Short communication

Changes in the mycovirus (LeV) titer and viral effect on the vegetative growth of the edible mushroom *Lentinula edodes*

Jung-Mi Kim[a], Ha-Yeon Song[a], Hyo-Jin Choi[a], Suk-Hyun Yun[b], Kum-Kang So[b], Han-Kyu Ko[c], Dae-Hyuk Kim[b,*]

[a] Department of Bio-Environmental Chemistry, Wonkwang University, Iksan, Chonbuk 570-749, South Korea
[b] Institute for Molecular Biology and Genetics, Center for Fungal Pathogenesis, Chonbuk National University, Jeonju, Chonbuk 561-756, South Korea
[c] Forest Mushroom Research Center, Yeoju, Gyeonggi 469-803, South Korea

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ABSTRACT

This study attempted to cure the edible mushroom *Lentinula edodes* strain FMRI0339 of the *L. edodes* mycovirus (LeV) in order to obtain an isogenic virus-free fungal strain as well as a virus-infected strain for comparison. Mycelial fragmentation, followed by being spread on a plate with serial dilutions resulted in a virus-free colony. Viral absence was confirmed with gel electrophoresis after dsRNA-specific virus purification, Northern blot analysis, and PCR using reverse transcriptase (RT-PCR). Once cured, all of fungal cultures remained virus-free over the next two years. Interestingly, the viral titer of LeV varied depending on the culture condition. The titer from the plate culture showed at least a 20-fold higher concentration than that grown in the liquid culture. However, the reduced virus titer in the liquid culture was recovered by transferring the mycelia to a plate containing the same medium. In addition, oxygen-depleted culture conditions resulted in a significant decrease of viral concentration, but not to the extent seen in the submerged liquid culture. Although no discernable phenotypic changes in colony morphology were observed, virus-cured strains showed significantly higher growth rates and mycelial mass than virus-infected strains. These results indicate that LeV infection has a deleterious effect on mycelial growth.

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The presence of viruses in fungi is now accepted as not "exceptional" but "general" (Dawe & Nuss, 2001; Ghabrial, 1998; Pearson et al., 2009) since the first definitive description of mycovirus more than 50 years ago (Hollings, 1962). Viral infection occurs in all major taxa belonging to the kingdom Fungi. However, it is difficult to determine the biological consequences of mycoviral infection for following reasons. First, although there is compelling evidence that an increasing number of viruses induce symptomatic infections, mycoviral infections, in general, have been considered not to cause measurable phenotypic changes in the fungal hosts. If anything, such changes are cryptic or latent. Second, in some cases, although mycoviral infection causes considerable morphological and physiological changes, including debilitation- and virulence-related phenotypes (Castro et al., 2003; Dawe & Nuss, 2001), the varied genetic backgrounds of fungal strains may respond differently to the same mycovirus, which makes it desirable to have identical genetic backgrounds (with the exception of the presence or absence of the virus) for the study of fungus–mycovirus interactions. Establishment of such virus-free and virus-infected isogenic lines to explore direct mycovirus–fungal host interactions has been hampered due to several reasons. Firstly, although several attempts have been described, successfully curing an infected fungal host of a mycovirus infection requires a great deal of meticulous endeavors (Aoki et al., 2009; Carroll & Wickner, 1995; Herrero & Zabalgogeazcoa, 2011; Romo et al., 2006; Souza et al., 2000). Secondly, although viral infection to virus-free strain via purified virion or RNA transfection is the most direct and simple way of horizontal virus transmission, the case is very limited. In addition, hyphal anastomosis, the most commonly known and probably the only naturally occurring mechanism of horizontal virus transmission, can accompany many cytoplasmic inheritances during the virus transmission. This makes it difficult to determine whether the changes in phenotypes are due to the mycovirus, other cytological inheritance, or both (Nuss, 2005).

The edible mushroom *Lentinula edodes* (Berk.) Pegler, commonly known as the shiitake, is the second most popular edible mushroom

* Correspondence to: Department of Molecular Biology, Chonbuk National University, Dukjindong 664-14, Jeonju, Chonbuk 561-756, South Korea.
Tel.: +82 63 270 3440; fax: +82 63 270 4312.
E-mail address: dhkim@jbnu.ac.kr (D.-H. Kim).

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in terms of worldwide production and economic value (Hadeler, 1995) and is one of two most important commercial mushrooms in Korea (Ko et al., 2012). Several reports on the discovery of mycovirus in *L. edodes* exist (Kim et al., 2013; Magae, 2012; Rytter et al., 1991; Won et al., 2013). However, because these mycoviruses are commonly found in healthy and asymptomatic fruiting bodies, it is unclear whether they are the direct cause of abnormalities (Rytter et al., 1991). In addition, it has not been determined if a relationship exists between the presence or absence of a particular mycovirus and the vegetative characteristics of mycelia.

In our initial survey of the abnormal browning of the medium surface and fruiting body of a popular commercial strain of *L. edodes* FMRI0339, we confirmed the presence of the mycovirus LeV (LeV-FMRI0339) (Kim et al., 2013) and demonstrated its meiotic stability. In the current study, we attempted to cure *L. edodes* FMRI0339 of LeV-FMRI0339 in order to establish virus-free and virus-infected isogenic lines. By pair-wise comparison of the isogenic lines, we sought to better understand the relationship between LeV infection and vegetative characteristics of *L. edodes*.

Based on the electrophoretic band pattern and Northern blot analysis (Park et al., 2008), the LeV concentration in *L. edodes* FMRI0339 varied significantly depending on whether the fungi were grown on a solid plate (Supplementary Fig. S1A–D; lane 1) or in submerged liquid culture (Supplementary Fig. S1A–D; lane 2). When the LeV-infected *L. edodes* FMRI0339 strain was grown in the potato dextrose broth (PDB) at 25 °C with continuous agitation (200 rpm) in darkness, the concentration of LeV from the same amount of mycelia was significantly reduced. More than a 20-fold decrease in the virus titer, as measured by ImageJ software, was observed from the mycelia submerged in a liquid culture (Supplementary Fig. S1A–D; lanes 1 and 2). When we tested different media, such as V8 juice, similar results were observed (data not shown). Together, these results indicate that the decreased viral titer is a result of growth conditions (e.g., solid vs. liquid), not media components. However, it was possible to isolate LeV from the limited amount (≥0.1 g of powder per preparation) of ground powder of lyophilized mycelia grown in liquid culture. Thus, no loss of virus from the preparation using mycelia grown in the submerged liquid culture was observed.

Interestingly, when the mycelia grown in liquid culture were transferred to the top of a plate containing the same medium, the freshly growing-mycelia on plate recovered a relatively high concentration of LeV (Supplementary Fig. S1A–D; lanes 3, 4, and 5). In addition, when the fungi were cultured by placing them gently on the surface of the liquid media without agitation, and were then used to prepare the virus from the floated mycelia, a high concentration of virus was maintained (Supplementary Fig. S1A–D; lanes 6, 7, and 8). These results indicate that growth conditions (e.g., submerged or not submerged) affect the viral replication significantly.

Since the submerged culture condition substantially decreased viral titer, we further examined viral titer under oxygen-limited conditions. For oxygen-depleted culture conditions, the Anaeropack-Anaero system (Mitsubishi Gas Chemical, Tokyo, Japan) was used to create a hypoxic environment in an air tight jar, as previously described (Van Horn et al., 1997). Cultures containing actively growing mycelia were placed in an air tight jar with the Anaeropack-Anaero system and incubated in darkness at 25 °C until use. Since the mycelial growth in an air tight jar with the Anaeropack-Anaero system was considerably decreased, we cultured for 20 days instead of 13 days in the air tight jar. No significant difference in the virus titer was observed between samples cultured for 13 days and 20 days on PDA. When the LeV-infected *L. edodes* FMRI0339 strain was inoculated on potato dextrose agar (PDA) and cultured for 20 days in an air tight jar using the Anaeropack-Anaero system, the concentration of LeV was significantly reduced (Supplementary Fig. S2A–D; lane 3). Densitometry indicated a two-fold decrease in the virus titer of the mycelia cultured under hypoxic conditions (Supplementary Fig. S2A–D; lane 1 vs. lane 3). Similarly, when the LeV-infected *L. edodes* FMRI0339 strain was cultured for 3 days in ambient air prior to transfer to hypoxic conditions, a two-fold decrease in the concentration of LeV was observed (Supplementary Fig. S2A–D; lane 1 vs. lane 4). These results indicated that the oxygen-depleted culture conditions affected LeV replication in fungal mycelia. However, considering the more than a 20-fold decrease observed in the submerged liquid culture compared to the two-fold decrease observed in the oxygen-limited condition, depletion of oxygen was not the only factor affecting viral titer in submerged liquid culture conditions. Further studies should examine what conditions other than hypoxia limit viral replication in fungal host.

Table 1
Growth rate and mycelial mass of virus-infected and virus-cured lines. Mean ± SD are shown with the range of measurement in parentheses.

	Growth rate (cm)	Mycelial mass (mg of dried weight)
Virus-infected *L. edodes* FMRI0339	6.95 ± 0.83[a*] (5.10–8.20)	11.73 ± 8.5[a] (2.2–44.6)
Virus-cured *L. edodes* FMRI0339-vf-1	7.42 ± 0.83[b] (6.40–8.40)	14.73 ± 11.4[a,b] (0.6–47.8)
Virus-cured *L. edodes* FMRI0339-vf-14	7.29 ± 0.75[b] (5.70–8.40)	17.67 ± 8.1[b] (1.9–36.3)

[*] Mean numbers followed by same letters in each column are not significantly different at the 5% level by *t*-test.

Viral titer reflects the host–parasite interaction. Besides differences in the genetic susceptibility of host, viral titer depends substantially on host cell physiology (Schoffelen et al., 2013) and culture conditions (Hillman et al., 1990; Jung et al., 2004). In nature, not only the genotypes of the host-parasite species but also environmental factors are important to select the outperforming interactions, which were hypothesized by the geographic mosaic theory of coevolution (Gomulkiewicz et al., 2000; Thompson, 1999). Accordingly, environmental factors were important to determine the outcome of the fungus–mycovirus interaction (Bryner and Rigling, 2011; Hyder et al., 2013). Thus, our study showing the variation in viral titer depending on culture conditions contributes to increase our understanding of the mechanism of variation in natural selection among ecosystems.

Substantial changes in LeV titer depending culture condition may explain why it was not easy to determine the outcomes of *L. edodes*–LeV interaction. Therefore, it is desirable to establish virus-free and virus-infected isogenic *L. edodes* lines to determine the effect of LeV infection on *L. edodes*.

Several methods have been applied to cure *L. edodes* FMRI0339 of LeV infection, such as hyphal tip transfer, cycloheximide treatment, incubation at low (20 °C) or high (30 °C) temperature, and mycelial fragmentation (Kim et al., 2013). Among these, the mycelial fragmentation method resulted in cured fungal lines. Based on the electrophoretic band pattern and Northern blot analysis of preparations of dsRNA by conventional CC41 cellulose chromatography, a high ratio (>40%) of colonies from mycelial fragments consisting of one to five cells lacked the LeV virus. This was further confirmed by RT-PCR using total nucleic acid and gene-specific primers corresponding to a part of RNA dependent RNA polymerase (RdRp) of the LeV in *L. edodes* FMRI0339 (GenBank Accession no. AB646992). The primers used were LeV-RDRP (forward) 5′-TTA TGG TCT GGA TGG CGT-3′ and LeV-RDRP (reverse) 5′-TGT CAC TCC AAA ACC TCC-3′. PCR was conducted as described previously (So et al., 2012). This simple mycelial fragmentation method of curing is extremely efficient and dependable as evidenced by repeated tests resulting in similar curing efficacy.

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