The Journal of Microbiology, February 2008, p. 95-99 Vol. 46, No. 1
DOI 10.1007/s12275-007-0171-y

The Identification of a Novel *Pleurotus ostreatus* dsRNA Virus and Determination of the Distribution of Viruses in Mushroom Spores

Yeo Jin Kim[1], Ji Yeon Kim[1], Ji Hye Kim[1], Seon Mee Yoon[2], Young-Bok Yoo[3], and Se Won Yie[1]*

[1]*Department of Molecular Bioscience, Division of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea*
[2]*Gyengggido Agricultural Research and Extension Services/ Mushroom Research Institute,*
[3]*National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon 441-707, Republic of Korea*

(Received September 21, 2007 / Accepted January 7, 2008)

Double-stranded RNAs and virus particles were identified in *Pleurotus ostreatus* strain Shin-Nong in Korea. Isometric virus particles with a diameter of 33 nm were purified, which are similar to other *Pleurotus* viruses reported previously. This strain contains 5 dsRNAs, 8.0, 2.5, 2.4, 2.0, and 1.8 kb in size. The virus particles contain 2 dsRNAs, designated RNA-1 (2.5 kb), and RNA-2 (2.4 kb) which is a typical pattern of *Partitiviridae*. A non-encapsidated dsRNA of about 8.0 kb also was identified. Partial cDNA from RNA-1 was cloned, and sequence analysis revealed that this gene codes for RdRp. The comparison of the sequence from partial cDNA clone showed 35% amino acid homology with the C-terminal end of the RdRp gene of *Helicobasidum mompa* virus and *Rosalinia necatrix* virus. Specific primers designed from the partial sequences successfully amplified RT-PCR product from the infected mycelium and a single spore culture. We used these primers to determine the pattern of distribution of viruses in spores. Of the 96 different single spore cultures generated from Shin-Nong strain, a specific RT-PCR product was identified in 25 cultures, indicating that about 26% of basidiospores contain viruses.

***Keywords*:** *Pleurotus* virus, dsRNA, fungal virus, virus distribution, cDNA

Double-stranded RNA fungal viruses have been reported in nearly all kinds of fungal genera including yeast and mushrooms. In general, fungal viruses are transmitted intracelluarly by hyphal anastomosis (horizontal transmission) and are believed to be disseminated via spores (vertical transmission). Some fungal viruses are associated with the hypovirulence of plant pathogenic fungi which include *Cryphonectria parasitica, Fusarium graminearum,* and *Rhyzoctonia solani*. (McCabe *et al.*, 1999; Liu *et al.,* 2003; Chu *et al.*, 2004). Some viruses are associated with the production of killer toxin which inhibits the growth of sensitive strains of the same genus. Killer toxin is usually coded by independent segment of dsRNA, which was studied extensively in *Saccharomyces cereviceae* (Schmitt and Breinig, 2002) and *Ustilago maydis* (Hwang and Yie, 1993). Other fungal viruses are also believed to be responsible for the abnormal growth of the host fungi, which is most important in mushroom industry. However, most of the fungal viruses do not cause any distinct symptom to their host, which are sometimes referred to cryptic viruses, or virus-like particles (VLPs). Based on the genome organization, phenotypic differences and virus structure, fungal viruses are classified into four different virus families, *Totiviridae*, *Partitiviridae*, *Hypoviridae,* and *Chrysoviridae* (Ghabrial, 1998). Members of *Totiviridae* usually contain single dsRNA coding for capsid and RNA-dependent RNA polymerase (RdRp), while *Partiviridae* contain two or more dsRNAs that code for capsid protein and RdRp, independently (Ghabrial, 1998). It was recently reported that many fungal species are infected with two or more different virus families together (Preisig *et al.*, 1998; Rong *et al.*, 2001). In other cases, additional dsRNA elements whose functions are not related to the virus replication, called satellite dsRNA elements, have been identified (Ghabrial, 1998).

Oyster mushroom (*P. ostreatus*) is the major product of Korean mushroom industry. Segmented dsRNA viruses have been identified in almost all the fungal species, including mushroom species used in cultivation industry all over the world. *Agaricus bisporus* virus (AbV) is considered to be the most important mushroom virus and is speculated to be a causative agent of La France disease (Romaine and Goodin, 2002). Encapsidated dsRNA mycoviruses were initially identified from diseased mushrooms (Hollings, 1962), and several other viruses have also been identified in *A. bisporus,* including the spherical or bacilliform particles of various sizes (Revill and Wright, 1997). Another mushroom disease called "virus X disease" was also recently reported, and it appears to be related to the presence of a dsRNA virus, called mushroom X virus (MXV) (Grogan *et al.*, 2003). A variable group of 26 novel dsRNA elements, ranging in sizes from 20.2 kb to 0.6 kb, was identified in both MXV-infected and even in healthy mushrooms. Several of these elements, approximately 17 of the 26 dsRNAs were reported to be non-encapsidated (Rao *et al.*, 2007). However, there has been no clear relationship between the presence

* To whom correspondence should be addressed.
(Tel) 82-33-250-8544; (Fax) 82-33-241-4627
(E-mail) swyie@mail.kangwon.ac.kr

of virus particle and a specific mushroom disease has been determined.

Two different genome types of mycoviruses were recently reported in *Pleurotus* species: one is referred to Oyster mushroom spherical virus (OMSV-1) containing single-stranded RNA that is strongly associated with epidemics of oyster mushroom Die-back disease (Yu *et al.*, 2003) and the other is called *Pleurotus ostreatus* virus-1 (PoV-1) and contains 2 segments of dsRNA and appeared to be a healthy strain in Korea (Lim *et al.*, 2005). The dsRNA virus of PoV-1 contains two dsRNA segments, one of which codes for capsid protein and the other codes for RdRp, which is a typical pattern seen in *Partitiviridae*. Many cultivars used in small- or large-scale mushroom farms and the standardization of cultivating strains just began only recently. Many different types of mycoviral dsRNAs are appeared to be infected in *Pleurotus* species. In this paper, we report that virus particles were isolated and a partial cDNA clone was obtained from *Pleurotus* strain Shin-Nong, designated PoV-SN. We also examined the distribution of the virus in the spore cultures by using a specific primer from the partial cDNA sequence.

Materials and Methods

Extraction of dsRNA

P. ostreatus strain Shin-Nong was used to extract dsRNA and purify the virus particles. Fungal mycelium was grown in a 5 L fermentor for 5 days and harvested with a cheese cloth. The mycelium was washed once with 0.1 M phosphate buffer (pH 7.0). Total nucleic acids were extracted with a homogenizer in the presence of 1 volume of phenol/chloroform and 1/2 volume of 1.0-mm glass beads. Total dsRNA was purified with a CF-11 column as described elsewhere (Morris and Dodds, 1979). The dsRNA was confirmed with DNase I and S1 nuclease treatment. The dsRNAs purified from CF-11 column and virus particles were analyzed on a 1.2% agarose gel and a 5% polyacryamide gel, respectively.

Synthesis of partial cDNA and sequence analysis

The dsRNA from the CF-11 column was physically sheared with Neubulizer (Invitrogen, USA) for 20 min in the presence of 2× shearing buffer (20 mM Tris; pH 7.0, 2 mM EDTA, 20% glycerol). Sheared dsRNA was denatured in boiling water for 10 min and the poly(A) tail was attached to the 3' end of sheared dsRNA with yeast poly(A) polymerase (Takara, Korea) according to the manufacturer's instruction. Tailed dsRNA was denatured as described above, and cDNA was synthesized with 200 U of reverse transcriptase (Takara, Korea) at 37°C for 1 h in the presence of 1 μmol of oligo-d(T) primer. Partial cDNA was cloned into pGEM T-vector (Invitrogen), followed by the sequencing of the inserts.

Purification and observation of virus particles

Fungal mycelium harvested from a 5 L fermentor was resuspended in 0.1 M phosphate buffer (pH 7.0) and homogenized with Bead-beater (Biospecs, USA) for 20 min in the presence of 0.5 mm glass beads. Cell debris was removed by high speed centrifugation at 13,000 rpm for 15 min, and virus particles were collected using a Beckman ultracentrifuge with a Ti70 rotor at 40,000 rpm for 2 h. Virus particles were further purified through 10~50% linear sucrose density gradient centrifugation as described previously (Hwang and Yie, 1993). The fraction containing virus particles was detected with the gradient fractionator (ISCO Inc., USA) and collected. Virus particles were stained with 0.1% uranyl acetate and observed with an electron microscope (LEO-912AB, Germany).

Northern blot analysis

RNA blot analysis was carried out as described elsewhere (Lim *et al.*, 2005). Briefly, the purified dsRNAs separated on 1.2% agarose gel were denatured by soaking in denaturing buffer. Denatured dsRNA was transferred onto a nylon membrane (Hybond, Amersham Bioscence, USA) with an electrotransfer in the presence of 2× SSC buffer. A DNA probe specific for RNA-1 was prepared by PCR amplification in the presence of biotin-labeled dCTP. The membrane was blocked with calf-thymus DNA, and hybridization was carried out at 50°C for 4 h followed by detection with Chemi-luminescence kit (New England BioLab, USA).

Isolation of monokaryotic strains

Basidiospores were obtained from spore prints of the mushroom fruiting bodies and suspended by serial dilution in distilled water. Spore suspension was plated in petri dishes containing approximately 20 ml of mushroom complete medium (MCM; Raper, 1972) and incubated for three to six days at 25~28°C. A single colony was transferred to a new MCM plate and confirmed as to be monokaryon by the observation of hyphae lacking clamp connection under the microscope.

RT-PCR analysis of monokaryotic strains

Monocaryons germinated from spores were grown on 50 mm culture dishes with MCM medium until the mycelium completely covered the dishes. The mycelium was collected by scraping with a scarfel and total nucleic acids were obtained by the extraction with phenol/chloroform using a mini-bead beater for 2 min in the presence of glass beads. RT-PCR was carried out with specific primers for dsRNA-1: SN-F; 5'-ATAGAATTCAAAAATACTTGTCATCCC-3' and SN-R; 5'-ATAGAATTCCTAAACAAATAGACGTTG-3'.
The PCR product was analyzed on a 1.5% agarose gel.

Results

dsRNAs from strain Shin-Nong

dsRNAs purified with CF-11 were analyzed by 1.2% agarose gel electrophoresis. Four distinctive dsRNAs, approximately 8.0 kb, 2.5 kb, 2.0 kb, and 1.8 kb in size, were clearly detected (Fig. 1A). Only 2.5 kb dsRNA band was observed on the agarose gel analysis of the sample directly extracted from the virus particles. When this dsRNA was further analyzed on a 5% polyaclylamide gel extensively, two dsRNAs were detected of less than 100 bp in size difference (Fig. 1B), indicating that these two dsRNAs are encapsidated in virus particles. These dsRNAs were designated RNA-1 and RNA-2, which may encode the RdRp and capsid protein, respectively, a typical pattern of the *Partitivirus* (Ghabrial,

1998). The 8.0 kb, 2.0 kb, and 1.8 kb dsRNAs were not detected in RNA sample obtained from the virus particles, suggesting that these two dsRNAs are not encapsidated in virus particles.

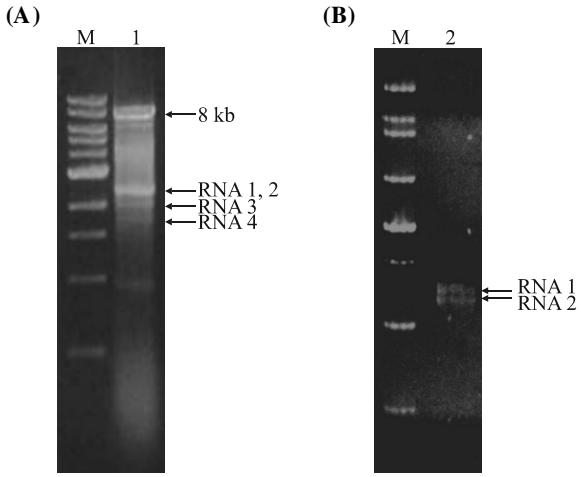

Fig. 1. Viral dsRNAs from *P. ostreatus* strain Shin-Nong purified with CF-11 were analyzed by 1.2% agarose gel electrophoresis (A). Viral dsRNAs extracted from virus particles were analyzed on 5% polyacryamide gel electrophoresis (B). Land M, 1 kb DNA ladder.

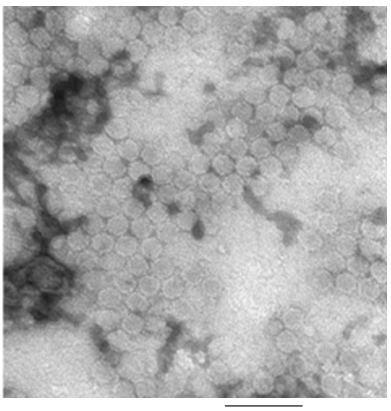

Fig. 2. Virus particles from *P. ostreatus* strain Shin-Nong. Virus particles were purified using a 10~50% sucrose density gradient and stained with 0.1% uranyl acetate. Bar represents 100 nm.

Virus particles

Virus particles purified by sucrose density gradient centrifugation were examined by electron microscopy. Virus particles of the Shin-Nong strain of the oyster mushroom had an isometric shape measuring 33 nm in size, which is consistent with the typical structure of *Partitivirus*-type mycovirus (Fig. 2). No other type of virus structure was observed in this preparation, which also indicates that the 8.0 kb dsRNA is not encapsidated with a protein capsid or may be enclosed with a membrane-type vesicles as described elsewhere (Romaine *et al.*, 1994). There were no structural differences among viruses from different species of *Pluerotus,* with the exception of the particle size.

cDNA cloning and partial sequence analysis

Partial cDNA synthesized from the sheared dsRNA was about 0.5 kb in size (data not shown). A cDNA was cloned into the pGEM-T vector, and the sequence was determined. There was no significant sequence homology with sequences in the GenBank data base. However, by the comparing of the amino acid sequence using NCBI Blast-X analysis, Shin-Nong strain partial cDNA showed 35% homology with the amino acid sequences in the C-terminal region of RdRp protein of *Partitivirus* isolated from *Helicobasidum mompa* (Osaki *et al.*, 2002) and *Rosellinia necatrix* (Sasaki *et al.*, 2005) (Fig. 3).

Specific primer

PCR was carried out in order to obtain 430 bp product using RNA-1 primers (Fig. 4). A probe generated by PCR using these primers was hybridized with the total dsRNA of the virus. Northern blot analysis showed that specific primer was successfully hybridized with RNA-1 indicating that RNA-1 is an RdRp gene.

RT-PCR of the single spore culture

Monocaryotic mycelium collected from the plate was tested for the presence of dsRNA-1 by RT-PCR in order to determine the distribution of virus on spores. A total 96 isolates were tested, and only 25 isolates were positive. RT-PCR was

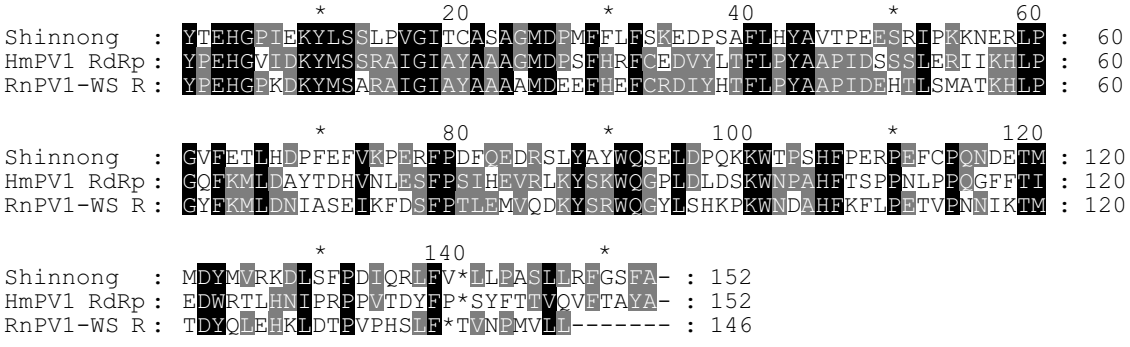

Fig. 3. Amino acid sequence alignment: Amino acid sequences from the partial cDNA of PoV-SN RNA-1 show homology with C-terminal end of *Helicobasidum mompa* virus and *Rosalinia necatrix* virus using NCBI Blast-X analysis.

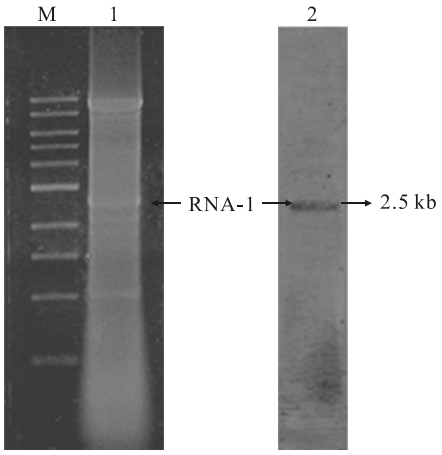

Fig. 4. Northern blot analysis of PoV-SN dsRNA-1. Labeled PCR product generated using primers from the partial sequences of RNA-1 specifically binds to viral dsRNA-1.

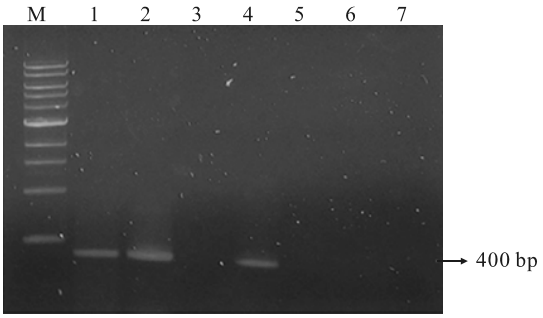

Fig. 5. PCR analysis of monocayon culture from the spores of *P. ostreatus* strain Shin-Nong. Lane 1, Dicaryotic strain Shin-Nong infected with PoV-SN virus; 2,4, Monokaryotic spore culture infected with a PoV-SN; 3,5,6,7, Virus-free monokaryotic spore culture.

repeated twice in an effort to reduce experimental error. This result indicates that only 26%, not all of the spores generated from virus-infected oyster mushroom contain viruses. No RT-PCR products were detected from samples directly extracted from a single spore or spore mixtures.

Discussion

No extracellular phases have been reported in fungal viruses, suggesting that viruses can be transmitted from cell to cell only by the cytoplasmic mixing such as hyphal anastomosis. This has been a major obstacle to testing the effects of virus on the host phenotype. There have been substantial evidences that the presence of viruses results in the reduction in mushroom yield or fruiting body malformation in *Agaricus* culture. However, there have been no clear evidences to suggest a relationship between the reduction of yield or abnormal growth of oyster mushroom and the presence of viruses. Numerous fungal viruses have recently been reported from all genera of fungi, including the oyster mushrooms (Yu *et al.*, 2003; Seo *et al.*, 2004; Lim *et al.*, 2005). Some of these whole or partial virus genes were cloned, and their sequences were reported. These sequence data have been used to analyze the presence of viruses in an *Agaricus bisporus* virus (Revill and Wright, 1997) and in an oyster mushroom strains in Korea (Kim *et al.*, 2005).

Based on the genome organization, two different families of *Pleurotus* virus have been reported, and they belong to single-stranded RNA virus (Yu *et al.*, 2003) and *Partitivirus* (Lim *et al.*, 2005). Therefore, it has been speculated that oyster mushrooms are mainly infected with *Partitviruses* and some other non-encapsidated large dsRNA elements as reported in this paper. To characterize the viruses isolated from *P. ostreatus*, cDNA cloning and the sequence analysis on the more oyster mushroom *Partitiviruses* are currently underway.

The effects of viruses on the oyster mushroom industry have been a focus of controversy for a long time. There are no clear evidences for a relationship between the presence of viruses and reduction of mushroom yield or malformation of a fruiting body yet. Nevertheless, the production of the virus-free strain has been a very important subject in mushroom industry. Various methods are used to generate virus-free strains. The generation of a protoplast from a hyphal tip was the most widely used method for the generation of virus-free strains. However, none of the methods are applicable to all industrially important mushroom species.

In this report we identified and characterized a novel isometric dsRNA virus with a genome containing 2 dsRNAs. The morphology of virus particles reported in this study was similar to those reported in other *Pleurotus* species (Lim *et al.*, 2005). The comparison of the sequence from the partial cDNA clone from RNA-1 showed homology with the C-terminal end of RdRp gene of *Helicobasidum mompa* virus and *Rosalinia necatrix* virus. Therefore, this virus is a novel *Pleurotus* virus, designated as PoV-SN. A specific primer designed from partial cDNA sequence was successfully used to test the presence of this virus in a mycelial culture and in a spore culture.

It has been believed that fungal viruses are vertically transmitted through spores. In *Aspergillus nidulans* and black *Aspergillus*, sexual spores and conidiospores are very efficient route for vertical transmission. Nearly all of the conidiospores were reported to contain viruses in *Aspergillus nidulans* (Van Diepeningen *et al.*, 2006) and in plant pathogenic hyphomycetes *Chalara elegans* (Park *et al.*, 2006). In the case of *H. annosum,* only 3 to 55% of conidiospores were reported to contain virus genomes, which indicates that the efficiency of vertical transmission of mycoviruses are different among spore types (Ihrmark *et al.*, 2002). Although there were no clear evidences, it has been speculated that the viruses can be transmitted via spore in oyster mushroom. Based on the results of RT-PCR experiment, we determined that only 26% of the spore cultures contain the RNA-1 segment. We could not explain the exact mechanism for the distribution of viruses on spores. It can be speculated that newly growing tissue may be present in the virus-free area, which results in uneven distribution of viruses during basidiospore formation. This is the first report to examine the distribution of viruses on *Pleurotus* spores. This result may be used to generate virus-free strains in the oyster mushroom industry. For example, virus-free strains will be manufactured

by the mating of the two different virus-free spore cultures.

Acknowledgements

This research was funded by grants from the Rural Development Administration. YJK, JYK, and JHK were supported by the graduate fellowship from the Ministry of Education through Brain Korea 21 Project.

References

Chu, Y.-M., W.-S. Lim, S.-J. Yea, J.-D. Cho, Y.-W. Lee, and K.-H. Kim. 2004. Complexity of dsRNA mycovirus isolated from *Fusarium graminearum*. *Virus Genes* 28, 135-143.

Ghabrial, S.A. 1998. Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* 16, 119-131.

Grogan, H.M., A.T. Adie, R.H. Gaze, M.P. Challen, and P.R. Mills. 2003. Double-stranded RNA elements associated with the MVX disease of *Agaricus bisporus*. *Mycol. Res.* 107, 147-154.

Hollings, M. 1962. Viruses associated with a die-back disease of cultivated mushroom. *Nature* 196, 962-964.

Hwang, S.H. and S.W. Yie. 1993. Genomic variation and toxin specificity of *Ustilago maydis* virus isolated in Korea. *Korean J. Microbiol.* 31, 184-188.

Ihrmark, K., H. Johannesson, E. Stenstron, and J. Stenlid. 2002. Transmission of double-stranded RNA in *Heterobasidium annosum*. *Fungal Genet. Biol.* 36, 147-154.

Kim, Y.-J., S. Park, S.W. Yie, and K.H. Kim. 2005. RT-PCR detection of dsRNA mycoviruses infecting *Pleurotus ostreatus* and *Agaricus blazei* Murrill. *Plant Pathol. J.* 21, 343-348.

Lim, W.-S., J.H. Jeong, R.-D. Jeong, Y.B. Yoo, S.W. Yie, and K.-H. Kim. 2005. Complete nucleotide sequence and genome organization of a dsRNA partitivirus infecting *Pleurotus ostriatus*. *Virus Res.* 108, 111-119.

Liu, C., D.K. Lakshman, and S.M. Tavantzis. 2003. Quinic acid induces hypovirulence and expression of a hypovirulence-associated double-stranded RNA in *Rhyzoctonia solani*. *Curr. Genet.* 43, 103-111.

McCabe, P.M., P. Pfeiffer, and N.K. Van Alfen. 1999. The influence of dsRNA viruses on the biology of plant pathogenic fungi. *Trends Microbiol.* 7, 377-381.

Morris, T.J. and J.A. Dodds. 1979. Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. *Phytophalology* 69, 854-858.

Osaki, H., K. Nomura, N. Matsumoto, and Y. Ohtsu. 2002. Characterization of double-stranded RNA elements in the violet root rot fungus *Helicobasidium mompa*. *Mycol. Res.* 108, 635-640.

Park, Y., X. Chen, and Z.K. Punja. 2006. Diversity, complexity and transmissin of double-stranded RNA elements in *Chalara elegans* (synanam. *Thielaviopsis basicola*). *Mycol. Res.* 110, 697-704.

Preisig, O., B.D. Wingfield, and M.J. Wingfield. 1998. Coinfection of fungal pathogen by two distinct double-stranded RNA viruses. *Virology* 252, 399-406.

Rao, J.R., D.W.A. Nelson, and S. McClean. 2007. The enigma of double-stranded RNA (dsRNA) associated with mushroom virus X(MVX). *Curr. Issues Mol. Biol.* 9, 103-122.

Raper, J.R. and C.A. Raper. 1972. Genetic analysis of the life cycle of *Agaricus bisporus. Mycologia* 64, 1088-1117.

Revill, P.A. and P.J. Wright. 1997. RT-PCR detection of dsRNAs associated with La France disease of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach. *J. Virol. Methods* 63, 17-26.

Romaine, C.P. and M.M. Goodin. 2002. Unravelling the viral complex associated with La France disease of the cultivated mushroom *Agaricus bisporus*, 237-257. *In* S.M. Tavantzis (ed.), dsRNA Genetic elements: Concepts and applications in agriculture, forestry and medicine. CRC Press, USA.

Romaine, C.P., B. Schlagnhaufer, and M.M. Goodin. 1994. Vesicle-associated double-stranded ribonucleic acid genetic elements in *Agaricus bisporus*. *Curr. Genet.* 25, 128-134.

Rong, R., S. Rao, S.W. Scott, and F.H. Tainer. 2001. Common multiple dsRNAs are present in populations of fungus *Discula destructiva* originating from widely separated geographic locations. *Curr. Microbiol.* 42, 144-148.

Sasaki, A., M. Miyanisi, K. Ozaki, M. Onoue, and K. Yoshida. 2005. Molecular characterization of a partitivirus from the plant pathogenic ascomycetes *Rosellina necatrix*. *Arch. Virol.* 150, 1069-1083.

Schmitt, M.F. and F.K. Breinig. 2002. The viral killer system in yeast: from molecular biology to application. *FEMS Microbiol. Rev.* 26, 257-276.

Seo, J.J., W.-S. Lim, J.H. Jeong, Y.B. Yoo, S.W. Yie, and K.-H. Kim. 2004. Characterization and RT-PCR detection of dsRNA mycovirus from Oyster Mushroom, *Pleurotus ostriatus*. *Plant Pathol. J.* 20, 200-205.

Van Diepeningen, A.D., A.J.M. Debets, and R.F. Hoekstra. 2006. Dynamics of dsRNA mycovirus in black *Aspergillus* populations. *Fungal Gen. Biol.* 43, 446-452.

Yu, H.J., D. Lim, and H.S. Lee. 2003. Characterization of a novel single-stranded RNA mycovirus in *Pleurotus ostreatus. Virology* 314, 9-15.