UDC: 632.38:577.213/.216:635.82

DIAGNOSIS AND PCR IDENTIFICATION OF MUSHROOMS MIKOVIRUS (MVX) (AGARICUS BISPORUS (J. LGE) IMBACH)

T. IVANOVA, Candidate of Agricultural Sciences,
M. MELNYCHUK, Doctor of Biological Sciences,
I. ANTIPOV, Candidate of Agricultural Sciences,
K. Grynchuk, graduate student

The features of infection with MVX of mushrooms in greenhouses in the Kyiv region are defined by visual diagnostics and electron microscopy methods. Symptoms of infection by virus X appears in brown colour of mushrooms fruiting bodies, darkness and lysis of mycelium, fruiting bodies deformation, wateriness of legs and caps, deformation and elongation of legs and extinction of caps. The role and place of the viral infection is proved. Using electron microscopy in samples of mushrooms X-Virus is discovered and bodies of rod shape of size ~ 70 nm is fixed.

First virologic monitoring of mycovirus prevalence in MVX of Kiev region is carried out. Of the 740 samples studied, in 87 were found symptoms of X-Virus mushrooms. Retrieved isolated dsRNK identified by PCR method, and their pathogenic properties are studied.

As a result of the analysis of dsRNK isolated from fruiting bodies of mushrooms the presence of viral infection is set. On the basis of conducted experiments it is recommended to use a sample of 10 g of starting material, the amount of STE buffer of primary washing 30 ml, adding 50% of phenol volume, 17 ml of chloroform and 2 ml of isoamyl alcohol. This method provides high-quality diagnosis and identification of RNA-containing viruses in microscopic and edible mushrooms.

***Keywords**: mushroom, virus, disease, identification, dsRNK, electron microscopy.*

Now in the world about 35 species of mushrooms, including 20 on an industrial scale are grown. [11]. Recently, Ukraine has increased production of cultivated edible mushrooms. Thus, in 2009, was produced 40 tons of mushrooms in 26 times more than in 2000 and almost 2 times higher than for example in Turkey [19].

Due to lack of reliable market information and official data on output mushrooms in Ukraine, these numbers are relative. By the volume of mushrooms production Kiev, Donetsk, Dnipropetrovsk, Odessa, Kharkiv and Lviv regions are preferred, in farms of which champignons and oyster mushrooms are grown [11.19], [4].

Scientists finally proved that almost every plant (as well as humans, animals, mushrooms, etc.) has its own specific viruses [6]. Viruses were divided in 73 fungal species belonging to 57 genus and five classes. Most of them are relatively avirulent [20].

Recently, by the application of simplified methods of diagnostic of mushrooms

diseases in farms where they are grown, the number of cases of detection of viral diseases and often from the first day of cultivation of mycelia [2].

Over the last 20 years in literature appeared the studies of viral diseases of champignons and its preventive measures [1-3,7-18]. The most dangerous among them is La France (LFIV or LIV), fruit bodies induced by virion size of 35 nm. This isometric virus was the subject of extensive epidemiological studies, so now La France disease is rare [2,8,9-11].

In the fruit bodies of mushrooms Ukrainian scientists found one virus A. Bisporus, which has a rod shape. Its length is 150-295 nm in diameter – about 18 nm. This pathogen reduced harvest of mushrooms throughout the harvesting cycle in 1,5-3 times.

During use of mechanical inoculation and injection of suspensions of sick mycelium and fruiting bodies, the pathogen can cause pathological changes even in some plants. In cells of inoculated plant virus is localized in the cytoplasm and is able to form intracellular inclusions. Established that it has antigenic relationship with tobacco mosaic virus [1,2,8].

After identification by M. Hollings of spherical (about 25-29 nm) and rod by morphology viruses (19x50 nm), appeared a number of reports that mushrooms are affected by viruses of different morphology. Conventionally, they can be divided into rod shaped, spherical and bacterial. This rod-shaped virus, according to all authors was of size about 270 17 nm, bacterial mostly – 19x50 nm and spherical – 25-45 nm. Interesting, that bacterial virus by its morphology is similar to alfalfa mosaic virus, but doesn’t have with him serological relationship [2,15].

In the conditions of natural biomes and industrial mushrooms production in Ukraine, mushroom viruses of about 32 nm, 18x52 nm and 150-295x 18 nm are isolated. It should be noted that in some studies bacterial and spherical virusus could not infect higher plants [2,4,15].

In recent years, viral diseases are very common in industrial production of mushrooms. In autumn 1996 in England was reported about reduction in yield of mushrooms, reasons of which were failed to install. Symptoms were absent, but the myceliums were virtually with no signs of fruiting bodies growth. From 1996 to 2003, the number of affected farms with similar etiology increased. Some symptoms responded the disease La France, but diagnostic tests of spherical virus showed negative results. Recently, the disease associated with the presence of new insulated double-stranded RNA (dsRNA) elements. However dsRNA differed from the previously described in A.bisporus and from dsRNA elements typical for the disease La France [9-11,13-15,17].

Was suggested that this disease was induced by non-descripted before virus with typical dsRNA genome. It was called "virus X", and later Mushroom Virus X (MVX) [8-10,13].

In 2010, several farms in Poland of growing mushrooms, reported about unusual brown mushrooms that appear on champignons plantations complexes. The symptoms are very similar to the MVX described by Irish scientists [13,14].

Foreign authors have suggested that the browning of mushrooms is caused not only by the presence of MVX, but could be caused by abiotic factors. There are data

that Pseudomonas bacteria can also cause these "stress effects" with similar change in morphology [14].

Thus, viral diseases not only reduce yield of mushrooms, but also lead to a total loss of mycelium. It is shown that after the viral disease, fruiting bodies of mushrooms vary morphologically, losing their taste qualities, have a reduced term of storage and often become unsafe for human consumption [2,11].

The greatest danger is to buy low-quality seed, contaminated with viruses [2]. Such viral mycelium grows slowly than healthy one. Today viral diseases of champignons that can affect fruiting bodies both in monoviroses and in various combinations (mixed viroses) [6]. External symptoms are visible to the naked eye on the fruit bodies could be various: oblong brown spots on legs, watery legs, browning and drying of pileus. Some of the symptoms are similar to the results of inadequate ventilation or excessive temperature: small pileus with long legs, quick breaking of tissues of the fruiting body. Often viral diseases of mushrooms are asymptomatic, resulting in reduced yield and quality of mushrooms [1,2].

The aim of our study was to develop a highly accurate and effective diagnostic test systems for the identification of champignons viruses, to provide rapid diagnosis and, consequently, highly accurate planning of laying plantations of mushrooms on virus-free basis. This will facilitate timeliness of conducting measures to prevent and combat with viral infections and will allow manufacturers to achieve good results in growing mushrooms.

Research methods. To study viral pathogens of mushrooms samples with specific symptoms and without them are used (Table).

Detection of viral diseases of champignons in Kyiv region

Study area of (spreading) viral diseases	External symptoms of fruiting bodies affection	Detection of viruses by the method of dsRNA selection	Affection, %, M± m
Kyiv, Vasyl'kivskyy district, Private Company	Dark brown spots, watery legs	+	8,5 ± 0,5
Kyiv Region, Kyevo-Svyatoshynskyy district, Private Company	Absent	+	7,0 ± 0,3
Kyiv Region, Vasil'kiv, Private Company	Dark fruiting bodies	+	6,3 ± 0,3
agro industrial complex, Kyiv	Brownish spots	+	1,6 ± 0,6

Experimental work was carried out during 2006-2011 on the base of the problem scientific-research laboratory of Phytovirology and biotechnology of National University of Life and Environmental Sciences of Ukraine. Some studies

were conducted in the scientific-research department of molecular-diagnostic researches of Ukrainian Laboratory of Quality and Safety of Products of Agro-industrial Complex, Laboratory of Mycelium Production SE NIO Agricultural Complex ‘Pushcha Vodytsya’ at mushroom private enterprises of Kyiv region.

To study viral pathogens of mushrooms samples with specific symptoms and without them were used. During the examination of the fruiting bodies of mushrooms in the mushroom farms of Vasyl’kivskyy, Kyevo-Svyatoshynskyy and Makarivskyy districts of Kyiv region and industrial mushroom enterprises of Kyiv in the period from 2006 to 2011 were selected 740 samples, in 87 of which were found symptoms of viral diseases. The objects of researches were fruiting bodies of champignons, compost, mycelium, roofing soil. For analyzes fruiting bodies with symptoms of disease were selected. In infected mushrooms during all waves fructification was observed. As control were fruiting bodies of champignons that were selected according to the results of visual assessment on damage and electronic-microscopic analysis in the laboratory conditions (Fig. 1).

Figure 1. Browning and lysis of fruiting bodies of champignons

For diagnosis and identification of unknown viruses, method of electronic microscopy and comfortable and previously tested method of isolation and identification of dsRNA, that was first proposed in the late 70th XX century, were used [5,6]. Excretion of dsRNA from mushroom fruit bodies was carried out by previously known methods [6,9,13], with some modifications [6,13].

Were taken 10 g of samples of mushroom fruit bodies, which were crushed and homogenized with the addition of liquid nitrogen in porcelain mortars. Homogenized samples were transferred into centrifuge tubes, added 12 ml of buffer 2x STE (H2O – 500 ml, NaCl – 29h, tris – 30,5 g, EDTA – 1,85 g), 1 ml of 1% SDS (H2O – 100 ml sodium dodecyl sulfate – 10 g) and 1 ml of bentonite, mixed with shaker for 15 minutes until the formation of indiscrete mass. Added 17 ml of STE-phenol (H2O – 500ml, 2x STE – 500 ml, pH 4.5) and 17 ml of chloroform-izoamil (24:1) and centrifuged for 20 min at 2500 r / min. After centrifugation, the aqueous phase was selected and then centrifuged for 10 min at 8000 rev / min [13], selected and added to 1.5 g of cellulose, 3 ml of absolute ethanol was stirred for 15-20 min. Then the

samples were transferred to ice for 30 minutes at a temperature of -15 º C. Content of tubes poured into column buffer STE-OH (STE – 100 ml ethanol – 174 ml, H2O – 726 ml) 20 ml and buffer STE – 20 ml. Filtrates were poured into centrifuge tubes and added 30 ml of ethanol, centrifuged for 30 min at 8000 rev / min [13], and poured on filter paper at a temperature of +18-20 º C – 2 hours, added 200 uL of 10X RNA buffer (0,35% (w/v) Orange G, 30% (w/v) Ficoll 400, 1 mM EDTA). In the resulting solution was added 1 ml of DNA-polymerase in the presence of 1 mm MgCl2 and transferred in the incubator for 1 h at a temperature of +37 º C.

Electrophoretic separation of nucleic acids dsRNA was carried out in 1% of agarose gel for 30 min at a voltage of 5-15 V/sm2 (Fig. 2). The remaining samples were stored at minus 20 º C.

Research results. During the electrophoretic separation was identified dsRNA fragment of size about 6 thousand pairs of nucleotide residues (W).

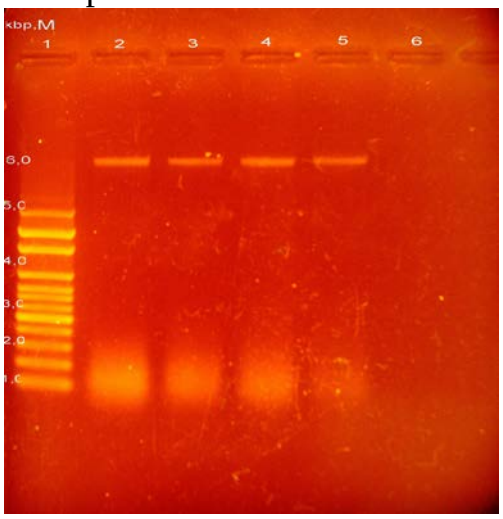

Figure 2. Electrophoregram (track 1 – DNA marker (Ladder DNA marker (100-5000 bp)), tracks 2-4 – samples of isolated dsRNA from the fruiting bodies of champignons, track 5 – sample of isolated dsRNA from asymptomatic fruit bodies of champignons)

As a result of analysis dsRNA isolated from fruiting bodies of champignons and electron microscopy the presence of a viral infection was established.

The presence of dsRNA fragments of viral origin coincides with the literature data on the possibility of mushrooms damage with specific viruses. The effectiveness of introduced methods of identification of dsRNA isolated from symptomatic and especially from asymptomatic fruit bodies of mushrooms can be argued. After many experiments, we recommend using during the analysis of each sample, 10 g of fungal mass for extraction of dsRNA. Our obtained data on the presence of viral dsRNA fragments are confirmed by electron microscopy.

Electronic-microscopic research of selected native material from each sample was performed by negative contrast method during fixation of preparation by 2% solution of phosphor volfram acid and revised on transmission electronic microscope EMV-ZA. Rod-shaped bodies of size ~ 70 nm were observed (Fig. 3).

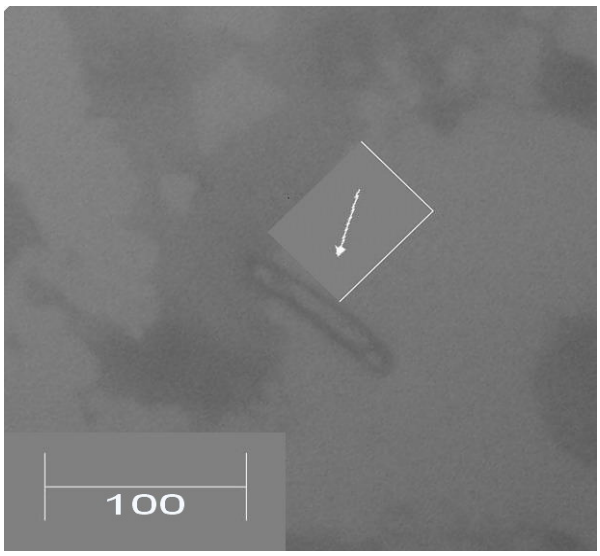

Figure 3. Electron diffraction of rod-shaped virus in champignons (Agaricus bisporus ((J. Lge) Imbach) Bar = 100 nm

Discovered on fruit bodies of champignons symptoms such as browning and lysis of fruiting bodies, elongation of legs (Fig. 1), allow us to talk about viral nature of their morphological destruction. Presence of dsRNA of viral origin in asymptomatic samples of mushrooms is observed.

Conclusions. As a result of researches on the fruiting bodies of mushrooms we identified dsRNA. The data of electronic microscopy confirmed the presence of viral infection.

Selected dsRNA fragments coincide with published data on the infection of mushrooms with specific mykoviruses of the family *Rhabdoviridae*.

Production recommendations. As a result of conducted researches, it is recommended to conduct required control of planting (maternal) material of mushrooms (mycelium) on the presence of viral pathogens. It is recommended for asymptomatic mushrooms too, which in turn, can be infected by virus in a latent (asymptomatic) form. The last one can be a hidden reason for the occurrence of mixed infections when during the infection by virus, mushroom cultures are susceptible to diseases caused by bacteria and other pathogens, resulting in loss of productivity and product quality. Approved system of diagnosis and identification of dsRNA fragment of size about 6 thousand pns. allows to conduct laboratory diagnosis to identify the rod-shaped virus of champignons (Agaricus bisporus) (J. Lge) Imbach).

References

1. Boyko O. Distribution, diagnosis and prevention boleznej shampynona dvusporovoho / O. Boyko, Melnychuk MD, Ivanova T. / / Dokl. Ross. Acad. s. – h. Science. – 2009. – № 2. – P.23-24.
2. Boyko O. Ecology and diagnosis of viral boleznej shampynonov / O. Boyko – K. Fytosotsyotsentr, 1999. – 24 p.
3. Devochkyn LA Mushroom / L.A.Devochkyn-Moscow: Kolos, 1975. – 112 p.

4. Dubinina A. Development of mushroom in Ukraine / A.Dubynina / / Food and processing industry. – 2009. – № 6-7. – P. 8-9.
5. Melnychuk MD Diagnosis and identification of RNA-containing fitovirusiv under their biosynthesis by examining the physical and chemical properties of double-stranded viral RNA / MD Melnychuk, R. Valverde / / Adv. Academy of Sciences of Ukraine. – 2001. – № 3. – P. 175-179.
6. Melnychuk MD Viruses of plants / MD Melnychuk Guide. – K. PolihrafKonsaltynh, 2005. – 200 p.
7. Methodological ukazanyya to ymmunodyahnostyke and prevention of viral boleznej shampynona / [IB Kaplan M.Talyanskyy, K. Alekseeva and others]. – Moscow: VASHNYL, 1986. – 22 p.
8. Vouko A. A. Diagnosis and prevention of virus – caused deseases of mushrooms in various ecological niches. / Vouko A. A. Tez. Proceedings. 1st international conference. Donetsk, October 1-4, 1997. – C.80-82.
9. Characterization of an RNA dependent RNA polymerase activity associated with La France isometric virus. J. / [Goodin M.M., Schlagnhaufer B., Weir T.,
10. Romaine C.P.] / / Virol. – 1997. – № 71. – P. 2264-2269.
11. Double-stranded RNA elements associated with the MVX disease of Agaricus bisporus / [Grogan HM, Adie BA, Gaze RH et al.] / / Mycol. Res. – 2003. – № 107 (2). – P. 147-154.
12. Elibuyuk I. Detection of a virus disease on Agaricus bisporus (white button mushroom) in Ankara, Turkey / Elibuyuk I., Bostan H. / / International journal of agriculture & biology. – 2010. – № 12. – P. 597-600.
13. Hollings M. Viruses associated with a die-back disease of cultivated mushroom / Hollings M. / / Nature – 1962. – № 196. – P. 962-968.
14. MVX disease and double-stranded RNA elements in Agaricus bisporus / [Grogan HM, Holcroft S., Adie B. Et al.] / / Mushroom Science. – 2004. – № 16. – P. 411-420.
15. Pudełko K. Mushroom virus X (MVX): a novel disease of mushrooms in Roland? / Pudełko K. / / Journal of plant protection research. – 2010. – Vol. 50, № 3. – P.366-371.
16. The wide distribution of endornaviruses, large double-stranded RNA replicons with plasmid-like properties / [Fukuhara T., Koga R., Aoki N.et al.] / / Archives of Virology. – 2006. – № 151. – P. 995-1002.
17. Transmission of Mushroom Virus X disease in crops / [Grogan HM, Tomprefa N., Mulcahy J. Et al.] / / Mushroom Science. – 2004. – № 16. – P. 489-498.
18. Wach M.P. Double-stranded RNA associated with La France disease of the commercial mushroom / Wach MP, Sriskantha A. and Romaine C.P. / / Phytopathology. – 1987. – № 77. – P.1321-1325.
19. Van Der Lende T.R., Harmsent M.C., Wessels J.G.H. Doublestranded RNAs and proteins associated with the 34 nm virus particles of the cultivated mushroom Agaricus bisporus. J. / / Gen. Virology. – 1994. – № 75. – P. 2533-2536.
20. http://www.golosua.com/ekonomika/2010/03/11/ For 9 years producing fungi in Ukraine increased by 26 times.
21. http://medbiol.ru/medbiol/sol_vir/ Viruses: rannye classification.

22. http://www.nbuv.gov.ua/portal/Chem_Biol/sgmb/2008_7/2008/SM07_17.pdf/ Kolomyjec L Characteristics of viruses that strike potatoes.