# ВІСНИК
## КИЇВСЬКОГО НАЦІОНАЛЬНОГО УНІВЕРСИТЕТУ ІМЕНІ ТАРАСА ШЕВЧЕНКА

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**Подано експериментальні дані про особливості будови, розвитку і функціонування рослинних і тваринних організмів, флору і фауну України, одержані на основі досліджень, що проводяться науковцями біологічного факультету в галузях фізіології рослин і тварин, генетики, ботаніки, зоології, мікробіології, вірусології. Викладено також нові дані стосовно біохімічних і біофізичних основ регуляції у клітинах і органах у нормі й після впливу різноманітних фізико-хімічних факторів, наведено результати нових методичних розробок.**

**Для наукових співробітників, викладачів, аспірантів і студентів.**

**Collection of articles written by the scientists of biological faculty contains data on research in molecular biology, physiology, genetics, microbiology, virology, botanics, zoology concerning the structure, development and function of the plant and animal organisms, flora and fauna of Ukraine. Results of newly developed biophysical methods of biological research, biochemical data regarding metabolic regulation under the influence of different factors are presented.**

**For scientists, professors, aspirants and students.**

# ЗМІСТ

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# STABILITY OF PLANT VIRUS GENOTYPE AND RISK ASSESSMENT FOR ARISING OF NOVEL VIRUS VARIANTS WITH ELEVATED EPIDEMIC POTENTIAL IN ANTHROPOGENICALLY TRANSFORMED ENVIRONMENT

***Данная работа посвящена моделированию продолжительного хронического воздействия тяжелых металлов, внесенных в грунт в нетоксичных концентрациях, на развитие вирусной инфекции системно пораженных растений. Нами продемонстрировано отсутствие статистически достоверных изменений в геноме ВТМ и подтверждена стабильность генома РНК-содержащих вирусов растений под хроническим воздействием абиотических факторов антропогенно трансформированной окружающей среды.***

***This work has been focused on modeling long-term chronic influence of heavy metals applied into soil in subtoxic concentrations on virus infection development in systemically infected plants. Here we have demonstrated the absence of statistically significant changes in TMV genome and confirmed high stability of the genome of RNA plant viruses under chronic effect of abiotic factors of anthropogenically transformed environment.***

**Introduction.** Chronic interplay among abiotic environmental factors and higher multicellular organisms and microorganisms including viruses is insufficiently explored. Following the examples of viruses, bacteria and plants it is known that intense short-lived effects of radioactive irradiation, UV irradiation, various chemical substances, temperature, etc. may induce genetic changes of mentioned organisms. However, the outcomes of prolonged chronic influence of abiotic environmental factors on plants and viruses are virtually unknown, – first of all, due to the difficulties in modeling such experiments. Meanwhile, the chronic type of interactions is the most important aspect in evolutionary terms, as it represents a powerful factor for organisms' variability [1].

In short, it has been established that heavy metal contamination of ecosystems favours plant virus spread [2, 3], more intense accumulation of viruses by systemically infected plants and delay in the onset of virus-specific symptoms [4]. We have also demonstrated positive correlation between the heavy metal content in soil and virus concentration in tissues of plants grown in such soil [5]. It has been revealed that bivalent cations of heavy metals may induce the appearance of the various aggregates of virus particles *in vi*tro [6]. Long-term virus passaging in heavy metal-stressed plants has been shown to affect neither virus infectivity nor the appearance of local virus-specific symptoms [5, 6].

According to the proposed hypothesis (partially confirmed by the outcomes of laboratory and small-scale field experiments), chronic effect of abiotic environmental stress factors leading to intensification of plant virus infection development may also contribute to genetic changes of RNA viruses of plants invoking further alterations of their biologic characteristics (in terms of the host range, symptoms' severity, pathogen's virulence). This, in turn, may be reflected at the population level in the form of more wide spread of virus infections in the ecosystems.

This work has been focused on modeling long-term chronic influence of heavy metals applied into soil in subtoxic concentrations on virus infection development in systemically infected plants for further assessment of probability of appearance/generation/prevalence of novel/mutated virus variants. Such variants may possess different biological characteristics posing certain threat in terms of elevated virulence or more severe symptoms, efficient spread, etc.

**Materials and methods.** In this work we have employed a well-studied model system "*Tobacco mosaic virus* – *Nicotianatabacum* cv. Samsun plants". Tobacco plants inoculation was done mechanically at the stage of four true leaves [7]. The following generations of experimental plants were inoculated using homogenate obtained from previous generations of plants of the respective group. Such procedure was repeated every time when inoculating plants. In such a way we have passed the virus three times (four generations of plants were used in total), and the duration of the experiment has totaled to 16 months. Plant samples from each generation were checked for virus presence and relative content via indirect ELISA [8] using specific anti-TMV polyclonal rabbit antisera. ELISA data (not shown here for the lack of space) confirmed stable virus transmission to the subsequent plant generations.

Further, we have analyzed possible molecular and evolutionary changes in virus genotype and the preservation of its genetic information to study the adaptation of the organisms to changing environmental conditions, as well as co-evolution of the parasite and its host using the 'plant-virus' model system under effect of anthropogenic stress factors.

Ten plants from the each experimental group were used for total RNA extraction using RNeasyPlantMinikit (Qiagen, UK) following the manufacturer's recommendations. RNA samples were further used for the RT-PCR amplification of cDNA (using specific primers and One-step-RT-PCR kit (Qiagen, UK) [9]) corresponding to the part of coat protein gene of *Tobacco mosaic virus* (TMV). Obtained RNA and cDNA preparations were checked using agarose gel electrophoresis [10]. Obtained cDNA preps have been sequenced and analyzed with bioinformatics packages (MEGA5 plus BioEdit) to see their mutual homology and reveal tentative differences in the sequence of CP gene of TMV induced by chronic influence of the heavy metals exerted on tobacco plants. The scheme of this experiment was generally adopted from Kearney et al. (1999) [11] and showed on Fig.1.

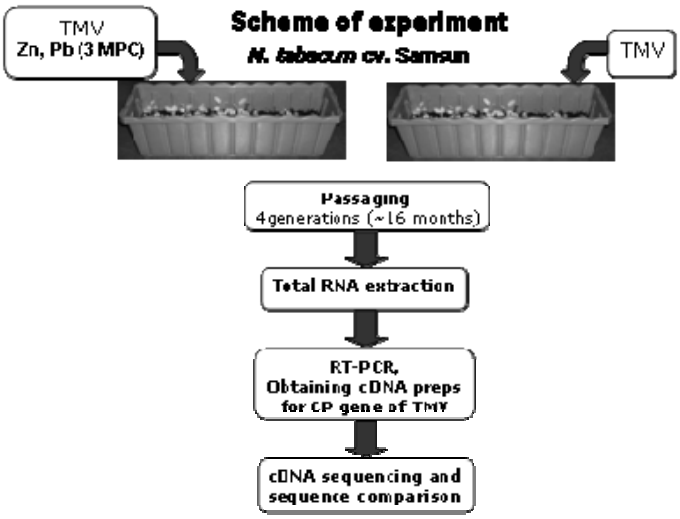


**Figure 1. The experiment algorithm for long-term passaging of TMV in tobacco plants subjected to chronic effect of heavy metals**

**Results and discussion.** Ten tissue samples of tobacco plants from each experimental group (two groups were continuously stressed with heavy metals for several generations, the other consisted of untreated virus-infected plants) were then subjected for total RNA extraction. Electrophoretic studies confirmed successful total RNA isolation from all groups of plants (Figure 2).

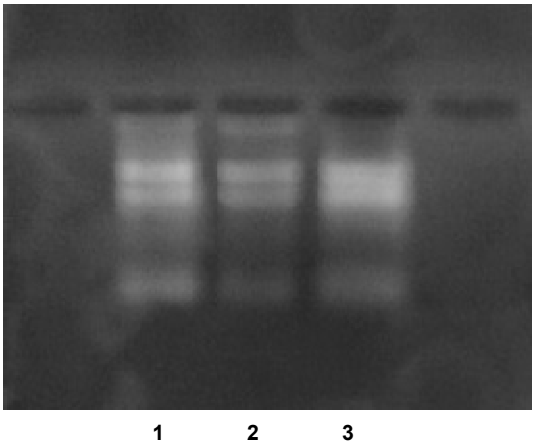


**Figure 2. Electrophoretic studies of total RNA preparations isolated using RNeasyPlantMinikit (Qiagen, UK) from:**
1 – TMV-infected tobacco plants not subjected to heavy metal stress ("control" plants);
2 – TMV-infected tobacco plants grown in Zn-amended soil;
3 – TMV-infected tobacco plants grown in Pb-amended soil

As can be seen from Figure 2, all obtained RNA preparations were comparable in yield and quality.

Further, total RNA preparations were used for the synthesis of corresponding cDNAs via RT-PCR using primers specific to the part of TMV U1 strain (known as reference or common strain and kindly provided by MSU, Russia). The analysis of obtained amplification products (Figure 3) confirmed that all total RNA samples (see Figure 2) (and hence, all respective plants) indeed contained recognizable (nucleotide sequence of) TMV. Obtained cDNAs were characterized with visually analogical and expected size in the gel (700 bp).

Although the technique in which the RT-PCR has been conducted cannot be considered as of quantitative nature, all measures were taken to ensure the uniformity of the samples' preparation and PCR itself. However, as can be seen from Figure 3, total RNA samples from metal-treated plants yielded more intense product band (tracks 2 and 3). The speculative nature of this argument nevertheless, has been directly confirmed by the outcomes of semiquantitative ELISA (Figure 4) demonstrating higher virus content in systemically invaded tissues of tobaccos subjected to heavy metal stress. This is in agreement with our previous results as for the same model system, as for TMV-infected tomatoes or PVX-infected potato plants [4, 12]. Hence, higher virus content in systemically infected host plants seems to be a general phenomenon when stressed with subtoxic concentrations of heavy metals.

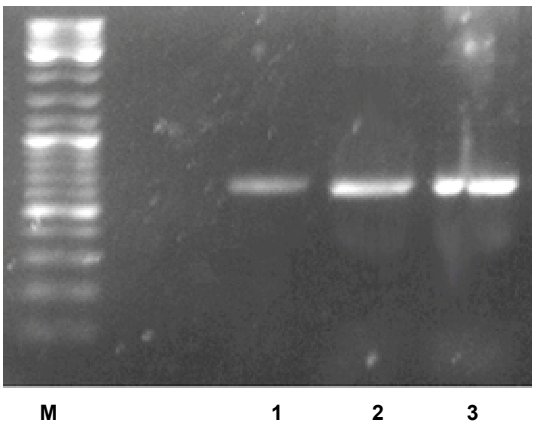


**Figure 3. Electrophoretic studies of cDNA preparations obtained (One-step-RT-PCR kit (Qiagen, UK)) with total RNA preparations isolated from:**
1 – TMV-infected tobacco plants not subjected to heavy metal stress ("control" plants);
2 – TMV-infected tobacco plants grown in Zn-amended soil; 3 – TMV-infected tobacco plants grown in Pb-amended soil

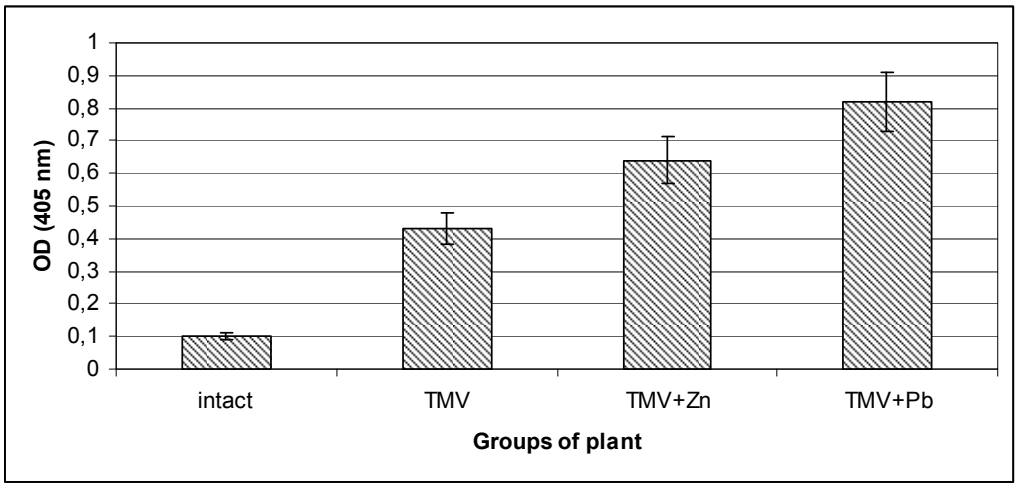


**Figure 4. Semiquantitative ELISA outcomes confirming efficient TMV replication in every plant group and showing higher virus content in tobacco plants grown in meal-enriched soil**

Further on we have carried out sequencing of the amplicons (see Figure 3) and their comparison. The outcomes have clearly shown nearly complete (99,9%) identity of cDNAs corresponding to the part of coat protein gene of TMV for all groups of experimental plants, including that subjected to long-term passaging under heavy metal stress (Figure 5).

Here we demonstrated the absence of statistically significant changes in the nucleotide sequence of CP gene of TMV under long-term chronic influence of heavy metals. Therefore, we have not confirmed the mutagenic effect of heavy metal in relation to TMV replicating in tobacco plants and have demonstrated high degree of evolutionary stability of RNA genome of the plant virus. In this regard we may suppose that heavy metals (at least zinc and lead, which are the most common metal pollutants found everywhere where human activity is conducted on a regular basis [1-4], and at least in these concentrations in soil) do not pose an appreciable threat in the context of sudden appearance of novel (possibly, more dangerous) isolates of plant viruses.

Of course, in this work we had several limitations among which: (1) single model system; but taking into account its wide use for various experiments, extremely wide spread (and significance) of TMV and that our previous works were mostly based on this system, we believe this choice is justified; (2) choice of heavy metals (discussed above); (3) duration of virus passaging in plants; this is the most speculative part of the story, as there is virtually no data to build upon; we deem that several generations of plants should have been enough for genetic changes (in the genome of TMV) to occur, should they appear. Considering the quasi-species nature of RNA viruses described for many of them including TMV, it is highly probable that novel virus variants may appear even under 'normal' conditions by that time, i.e. without any effects induced by additional stressors. In such case we would presumably see slight differences in one of the less conservative coat protein gene of TMV. As this is not the case, another reason may lay with the primers designed to detect conservative part of the CP gene of TMV. Should the mutations appear in a different part of the gene, it would pass unnoticed. This is taken into consideration and will be the subject for further work.

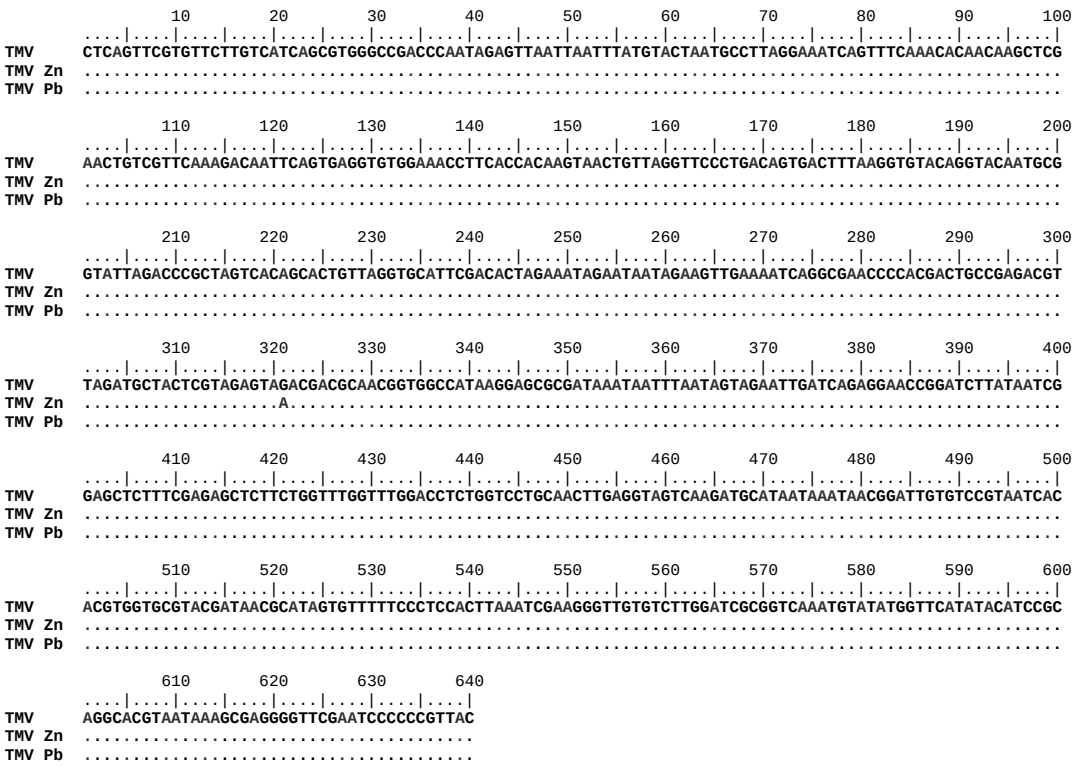


**Figure 5. Comparison of sequences of cDNAs corresponding to the part of coat protein gene of TMV (aligned using MEGA 5 package and processed using BioEdit package) for different plant groups (TMV – TMV-infected plants not treated with heavy metals; TMVZn – TMV-infected plants growni nZn-amended soil; TMVPb – TMV-infected plants grownin Pb-amended soil)**

**Conclusions.** In this work we have demonstrated the absence of statistically significant changes in the part of CP gene of TMV after long-term passaging in systemically infected host plants, *Nicotianatabacum*cv. Samsun, and have not demonstrated the mutagenic effect of heavy metals towards TMV.

Using TMV as a model virus we have confirmed high stability of the genome of RNA viruses under chronic effect of abiotic factors of anthropogenically transformed environment.

Considering the obtained experimental outcomes it is deemed highly unlikely that there is a real danger for the random and unexpected appearance of novel plant virus strains in anthropogenically stressed environment (taking into account the limitations above).

**Acknowledgments.** The authors are indebted to Dr. Tetyana Shevchenko (Department of Virology, Taras Shevchenko' Kyiv National University) for providing specific anti-TMV polyclonal rabbit antiserum and to Dr. Ivan Boubriak (Biochemistry Department, Oxford University) for his invaluable help with sequencing.

1. Dmitriev A., Shevchenko O., Polischuk V., and Guscha N. 2009. Effects of low dose chronic radiation and heavy metals on plants and their fungal and virus infection. Data Science Journal, 8(24): 49–57. 2. Polischuk V.P. 2005. Effects of radioactive and chemical pollution on plant virus frequency / V.P. Polischuk, T.P. Shevchenko, I.G.Budzanivska [et al]. NATO Security through Science Series, Environmental Security: Vol.2., Equidosimetry (F. Brechignac and G. Desmet, eds.). P.87-92. 3. Polischuk V.P., Budzanivska I.G., Shevchenko T.P. Abiotic environmental factors: effects on epidemiology of plant virus infections Cooper J.I. et al. (eds.).2006. Virus Diseases and Crop Biosecurity. NATO Security through Science Series, Springer. P.121-132. 4. ShevchenkoO., KamzelO., BudzanivskaI., ShevchenkoT., SpaarD. and PolischukV. 2006. Heavy metal soil contamination delays the appearance of virus-induced symptoms on potato but favours virus accumulation. Archives of Phytopathology and Plant Protection, 40(6): 406-413. 5. Kamzel O.A., Petrenko S.M., Budzanivska I.G., Shevchenko O.V. 2007. Correlation between the zinc content and TMV replication efficiency in tobacco plants. The Bulletin of Taras Shevchenko' Kyiv National University. Biology Series, 49-50: 100-101. 6. Kamze lO.A., Litvintsova O.V., Shevchenko O.V., Boyko A.L. 2008. EffectofzincandleadionsofIn vitro aggregationofTMVparticles. Agroecology Journal, Spec. Issue: 88-89. 7. Hill S.A. Methods in plant virology. Oxford: Alden Press, 1984. 167 p. 8. Crowther J.R. ELISA. Theory and practice. N.Y.: Humana Press, 1995. 96 p. 9. QIAGEN$^{R}$. One StepRT-PCRKitHandbook. Quiagen, 2002. 39 p. 10. Sambrook J., Fritsch E.F., Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY, 1989. Vol. 1, 2, 3. 11. Kearney C.M. M.J. Thomson, K.E. Roland. 1999. Genome evolution of tobacco mosaic virus population during long-term passaging in diverse range of hosts, 144: 1513-1526. 12. Shevchenko O.V., Budzanivska I.G., Shevchenko T.P., Polischuk V.P. 2004. The course of virus infection of Lycopersicon esculentumplants subjected to high concentrations of heavy metals. Microbiology Journal, 66(3): 80-88.

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O. Ivashchenko

# THE SPREAD OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS IN UKRAINE

***В статье приведены результаты исследований по выявлению антител к вирусу репродуктивного и респираторного синдрома свиней (ВРРСС) из 23 областей Украины и АР Крым. Результаты исследования свидетельствуют о циркуляции ВРРСС в 28 хозяйствах 19-ти областей Украины. Обнаружено тенденцию распространения ВРРСС на территории Украины по сравнению с исследованиями эпизоотической ситуации 2006-2007 годов.***

***The results of this study concern the detection of antibodies against PRRSV in serum from 23 regions of Ukraine and the Crimea. The results indicate porcine reproductive and respiratory virus (PRRSSV) circulation in 28 farms from 19 regions of Ukraine. The end results show trends of spreading PRRSV in Ukraine compared with studies of epizootic situation in 2006-2007.***

Porcine reproductive and respiratory syndrome virus are currently prevalent in most pork producing countries. It's one of the main causes of severe reproductive disorders in swine and respiratory disease complex [1,2]. Currently, due to losses associated with these reproductive and respiratory problem in pigs PRRSV is considered to bean economically importantviral pathogen in the pork industry [2]. This contagious disease of pigs of different ages may manifest acute or subclinical [3].

PRRSV is a member of the family *Artereviridae* in the order *Nidovirales.* Sequencing and antigenic properties showed existent of two distinct genotypes Europinian (type 1) and North-American (type 2) [2]. The two genotypes shared only about 63% nucleotide identity on the genome level. The evolution rate of PRRSV fixed as the highest among RNA viruses [5] and it is one of the causes of complexity in diagnostics, vaccination and controlling PRRSV.

From the first time PRRSV was detected in Canada in 1979, it rapidly spread in North America in the late 80s. Genetically different PRRSV, with similar clinical occurrence, appeared in Europe and spread through swine-growing regions during 1990-1992 [1, 6].

For more than10 years, PRRSV spread in most pig populations. Currently the virus is recorded in the majority of pork-producer countries. PRRSV was not found only in Australia, New Zealand, Norway, Finland, Sweden and Switzerland [1].

Clinical manifestations of PRRSV vary depending on the virulence of the virus, the immunestatus of the herd, and the age of infected animals. The disease, which clinically manifests, is a result of viremia. Ability of PRRSV to overcome transplacental barrier and infect the fetus causes abortions in sows and births of weak, nonviable piglets [1, 4].

Most of the clinical manifestations associated with PRRSV infection were investigated by experimental infection. Field observations of PRRSV infection in combination with other viral and bacterial agents can modify and complicate the clinical manifestations of disease [3, 7, 8]. The similarity of clinical signs of disease and diversity of reproductive diseases of pigs complicates setting even priordiagnosis. It is necessary to use laboratory diagnostic methods for the diagnosis of infection of PRRSV in infected animals. ELISA is simple in works, fast, reliable enough, with a high specificity method. All these advantages make ELISA one of the methods of choice for the diagnosis of PRRSV.

The epizootic situation in Ukraine of PRRSV was studied not enough and that was why our main aim was to study the spread of the porcine reproductive and respiratory syndrome virus in Ukraine.

**Materials and Methods.** For detection of antibodies to PRRSV, 108 herds were selected on the basis of clinical signs. This selection was based on information of the herd veterinarian. In all herds, the sampling of serum samples from animals of different technological group was carried out. Samples of serum were taken from the main herd animals with the clinical science of PRRSV and from boars in these farms. Biological material was delivered in thermal containers with ice. The volume of each sample was 3ml. It also recorded the necessary information: clinical science, animal age, name of the farm, sector, region. For this study 7,937 serum samples were collected from animals from 23 regions of Ukraine.

Sera were tested for the presence of PRRSV antibodies by ELISA (IDEXX HerdCheck PRRS 2XR ELISA, USA). Overall procedure was performed according to manufacturer's manual.

Samples of serum were collected 10-14 days after the appearance of first clinical signs [4, 9]. The presence of maternal antibodies to PRRSV and the fact of their decreasing to 9 weeks of animal life were also taken into account [4, 10].To obtain reliable results, serum samples were collected from animals of different technological groups from one farm [9] in an amount not less than 12 [4].

**Results and their Discussion.** Currently PRRSV was found in most pork producing countries. The genetic material of the PRRSV was detected in France, Germany, Belgium, Denmark, Spain, Italy, Czech Republic, Latvia, Lithuania, Russia, Belarus, Kazakhstan, Ukraine, China, Japan, Vietnam, Thailand, and the United States [11, 12, 13, 14].

In 2006-2007, Havrasyeva et.al and others studied the distribution PRRSV in 16 regions of Ukraine, the circulation of the virus was detected in 12 of them [15]. It should be noted that in 2007 VRRSS not diagnosed in animals of Ternopil and Chernihiv regions, while in 2011 the PRRSV positive animals in these regions were found. The results of Havrasyeva et.al. in the Dnipropetrovsk region from 2006 showed 1.25% of PRRSV positive animals by ELISA. The increase of the number of PRRSV infected animals were observed (7, 5%) in 2007 [15]. In 2011, we recorded PRRSV infection in 39% of the animals. It indicated the spread of the PRRSV through Ukraine.

In 2006, PRRSV was diagnosed in 41.3% of animals from Kyiv region, whereas in 2007 no serologically positive PRRSV animals were found in this region [15]. These results can be explained by selection oft he blood serum samples from animal of different farms in the Kyiv region, in sufficient sample size in 2007, or different age groups of animals that were studied. In 2011, we studied nine farms from the region around Kyiv and detected 30,5% of serologically positive for PRRSV animals. However, this data may no treflect that the reduction of circulating virus in the region was due to significan tdifferences in sample size. This is different from the way the serum was analyzed from two farms of this region in 2006, compared to the 9s in 2011.

It is also necessary to emphasize the epizootic situation stability in the Kherson region and in the Crimea. The antibodies against PRRSV were not detected in serum samples from this region in 2007, 2011 years [15].In general, we have analyzed 7937 serum samples from 108 farms in 23 regions of Ukraine and Crimea. 1265 serum samples were serologicaly PRRSSV positive (Table1).The circulation of the virus detected in 21 regions of Ukraine (Table 1., Fig. 1).Our results significantly complete the existing information about spreading PRRSSV and indicate the trend of spreading the pathogen in the farms of Ukraine. PRRSSV

circulation was found in 28 herds, while the situation of the disease in 4 farms remain uncertain. It requires additional studies and further monitoring of blood serum for the presence of increasing or decreasing the level of antibodies to PRRSV and determine the number of PRRSSV positive animals in these farms (Table.1).

***Table* 1. The results of detection PRRSSV antibodies in serum samples**

| № | Region | No. of farms | No. of seropositive farms | No. of samples | No. of seropositiv samples | No. of Seropositiv samples % |
|---|---|---|---|---|---|---|
| 1 | Vinnitsa | 6 | 2 | 46 | 16 | 34, 8 |
| 2 | Volyn | 2 | 1 | 110 | 6 | 5, 5 |
| 3 | Dnypropetrovsk | 15 | 3 | 410 | 160 | 39 |
| 4 | Donetsk | 10 | 3 | 754 | 314 | 41, 6 |
| 5 | Zhytomyr | 3 | 1 | 52 | 41 | 78, 8 |
| 6 | Zaporyzha | 8 | 4 | 4798 | 263 | 5, 5 |
| 7 | Zakarpatska | 4 | 2 | 88 | 25 | 28, 4 |
| 8 | Kyiv | 9 | 1 | 315 | 45 | 30, 6 |
| 9 | Kirovohrad | 3 | 1 | 147 | 24 | 16, 3 |
| 10 | Lugansk | 1 | 0 | 6 | 0 | 0* |
| 11 | Lvyv | 4 | 2 | 99 | 61 | 61, 6 |
| 12 | Mucolayv | 1 | 0 | 10 | 0 | 0* |
| 13 | Odessa | 3 | 1 | 57 | 7 | 12, 3 |
| 14 | Poltava* | 3 | 1 | 146 | 1 | 0,7* |
| 15 | Rivne | 1 | 0 | 12 | 0 | 0 |
| 16 | Sumy | 4 | 2 | 176 | 78 | 44, 3 |
| 17 | Ternopil* | 2 | 1 | 29 | 1 | 3, 4* |
| 18 | Kharkiv | 4 | 1 | 193 | 129 | 66, 8 |
| 19 | Kherson | 3 | 0 | 39 | 0 | 0 |
| 20 | Khmelnytsky | 3 | 2 | 58 | 46 | 79, 3 |
| 21 | Cherkasy | 8 | 2 | 99 | 10 | 10, 1 |
| 22 | Chernivtsi | 4 | 1 | 25 | 4 | 16 |
| 23 | Chernihiv | 6 | 1 | 92 | 33 | 30,3 |
| 24 | Crimea | 2 | 0 | 103 | 0 | 0 |
| Total | 23 regions and Crimea | 109 | 32 | 7937 | 1265 | |

* regions requires further research to establish the epizootic situation based on a small number of serologically positive animals


**Fig.1. The spread of the porcine reproductive and respiratory syndrome virus in Ukraine**

– PRRSV circulation were detected

– No PRRSV circulation were detected

– areas that require further research to establish the epizootic situation

– areas that weren't studied

– areas that were studied in 2006-2007 [15]

With this data we can come to a conclusion about the circulation of the virus in 19 regions of Ukraine. In the herds of Poltava and Ternopol regions, there was detected one PRRSV positive serum. We believe more research is needed to monitor dynamic changes of PRRSV antibodies in serum for the establishment of the epizootic situation in the farms in these areas. PRRSV was not detected only in herds from Lugansk, Rivne, Kherson regions, and AR Crimea (Table1). However, it should be noted that the number of samples that we examined in Lugansk, Rivne and Kherson regions were insufficient for analysis of epizootic situation and therefore, more research is needed.

Within a herd, PRRSV can rapidly spread through nose-to-nose contact and inutero infections [3, 12]. There are a number of factors to the contribution of PRRSV such as animal welfare, increasing herd size, carriers from purchased animals, and sperm [3, 12]. Infection can also be spread through PRRSV-contaminated clothing via injection [3]. Active animal trade, the purchase of pigs, and semen from different farms in Ukraine and the world, non-technology holding of animals, and a high variability of the pathogen can cause the spread PRRSV in Ukraine.

**Conclusions and Prospects for Further Research.** 1. The results suggest the circulation of the porcine reproductive and respiratory syndrome virus in 28 farms in 19 regions of Ukraine. 2. The results significantly complete the existing information about spreading PRRSV and indicating the spreading trend of the pathogen through Ukrainein comparison with studies epizootic situation 2006-2007. 3. It is important to create a bank of PRRSV isolates to make their sequencing for further analysis of the situation with PRRSV in Ukraine.

1. Kim H.K., Yang J.S., Moon H.J. et al. Genetic analysis of ORF5 of recent Korean porcine reproductive and respiratory syndrome virus (PRRSVs) in viremic sera collected from MLV-vaccinating or non-vaccinating farms // Journal of Veterinary Science. – 2009. – Vol.10. – P.121-133. 2. Van Breedam W., Costers S., Vanhee M. et. al. Porcine reproductive and respiratory syndrome virus (PRRSV) – specific mAbs: supporting diagnostics and providing new insights into the antigenic properties of the virus // Veterinary Immunology and Immunopathology. – 2011. – P.1-12. 3. Rownald R.R.R. The interaction between PRRSV and the late gestation pig fetus// Virus Research – 2010. – Vol. 154 – P.114-122. 4. Duinhof T.F., Schaik G. van, Esch E.J.B. van, Wellenberg G.J. Detection of PRRSV circulation in herds without clinical signs of PRRSV: Comparison of five age groups to assess the preferred age group and sample siza // Veterinary Microbiology. – 2001. – Vol.150 – P. 180-184. 5. Forsberg R. Divergense time of porcine reproductive and respiratory syndrome virus subtypes// Mol. Boil. Evol. – 2005. – Vol 2. – P. 2131-2134. 6. Amonsing A., Kedkovid R., Puranaveja S. et al. Comparative analysis of complete nucleotide sequence of porcine reproductive and respiratory syndrome Virus (PRRSV) isolates in Thailand (US and EU genotypes) // Virology Journal. – 2009. – Vol.6. – P.280-289. 7. Инфекционные болезни животных / Б.Ф. Бессарабов, Е.С. Воронин и др.; Под ред. А.А. Сидорчука. – М.: КолосС, 2007. – 671 с. 8. World organization for modern health. PRRS: the disease, its diagnosis, prevention and control // Report of the OIE AD HOC group on reproductiveandrespiratorysyndrome. – Paris.-2004. 9. Ladinig A., Balka G., Weissenboeck H., Saalmueller A., Gerner W., Kaeser T., Rusvai M., Ritzmann M. PRRSV (Type I) challenge in finishing pigs // International symposium on emerging and re-emerging pig diseases, 2011. 10. Kim S.H., Roh I.S., Choi E.J., Lee C., Lee C.H., Lee K.H., Lee K.K., Song Y.K., Lee O.S., Park C.K. A molecular analysis of European porcine reproductive and respiratory syndrome virus isolated in south Korea// Vet Microbiol. – 2010. – Vol. 143. – P. 394-400. 11. Stankevicius A., Cepulis R., Zilinskas H., Stadejek T., Pejsak Z. Persistence of European and American type PRRSV strains within Lithuanian pig herd // Bull. Vet. Inst. Pulavy. – 2008. – Vol.52. – P.319-323. 12. Murtaugh M.P., Stadejek T., Abrahante J.E., Lam T.T.Y., Leung F. C-C. The ever-expanding diversity of porcine reproductive and respiratory syndrome virus// Vir. Res. – 2010. – Vol.154. – P.18-30. 13. Karniychuk U.U., Geldhof M., Vanhee M., Van Doorsselaere J., Saveleva T.A., Nauwynck H.J. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate//BMC Vet. Res. – 2010. – Vol.6. – P.30. 14. Shi M., Lam T.T.Y., Hon C-C., Hui R.K.H., Faaberg K.S., Wennblom T., Murtaugh M.P., Stadejek T., Leung F.C.C. Molecular epidemiology of PRRSV: A phylogenetic perspective//Vir. Res. – 2010. – Vol.154. – P.7-17. 15. Гаврасьєва Н.В., Головко А.М., Троценко З.Р., Абрамов А.В. Ретроспективна діагностика репродуктивного та респіраторного синдрому свиней методом імуноферментного аналізу// Ветеринана біотехнологія. – 2008. – №13.

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## SELECTION OF BACTERIOPHAGES, SPECIFIC TO *SERRATIA MARCESCENS*

***Представлены результаты скрининга бактериофагов специфичных к возбудителю гнили лука. Высокостабильный литический фаг, специфичный к S. marcescens выделенный из окружающей среды и обозначен как бактериофаг Smd. У выделенного фага изучена морфология вирионов и состав белков. Показана способность фага Smd1 подавлять развитие бактериальной инфекции на чешуйках лука.***

***The results of screening bacteriophages specific to causative agent of onion decay are presented. H***ighlystable lytic phage specific for ***S.marcescens was isolated from the environment and denoted as phage Smd1. The virion morphology and protein content of isolated bacteriophage was studied. There was shown the ability of phage isolate Smd1 to reduce the development of bacterial infection on an onion scales.***

**Introduction.**Modern society focuses on ecologically pure future. Guarantees of that are "naturalization" of production, management of natural resources and energy.The preservation of crop losses caused by bacterial pathogens is significant problem for Ukraine and international community. Nowadays pesticides and herbicides, that in chemical nature are antibiotic substances, are widely used for prophylactic and therapeutic purposes. Only the last 20-30-years scientists began to pay special attention to the processes of resistance formation of pathogenic bacteria to the antibiotics. Therefore people faced the problem of search and development of new protective forms that would be characterized by high efficiency, safety and would not cause rapid adaptation of bacteria. The hypothesis of the possibility of using bacteriophages (viruses of bacteria) for therapeutic purposes was nominated in the beginning of the $20^{th}$century. Nowadays, this direction develops rapidly and expands the range of phage usage [2,3]. Significant development as acquired agricultural phage therapy that is engaged in elaboration of phage preparations to deal with pathogenic bacteria (for example, *Erwinia carotovora*, *Pseudomonas sp.*, *Serratia marcescens*).

Due to the importance and attractiveness of the area of research, the aim of our study was the selection and characterization of lytic phages, specific to the pathogen *Serratia marcescens*, which causes an onion decay and is considered to be one of the most harmful phytopathogens for agriculture in Ukraine.

**Materials and methods.**

**Bacterial strains.**Studies were carried out on bacterial culture *Serratia marcescens* IMBG291 [5], generously provided by colleagues from the laboratory of microbial ecology, Institute of Molecular Biology and Genetics (National Academy of Sciences of Ukraine). Working with phages we used an overnight culture of bacteria, in which bacteria was in exponential phase of growth. The concentration of bacteria cell culture was $10^8$-$10^9$ c.f.u./ml. Bacteria was cultivated on plate count agar or in PC-broth. Incubation temperature was 25 $^{O}$C.

**Sample collection.**During laboratory studies a wide range of samples was analyzed. Samples were selected from vegetable washouts (including onion, potatoes, cab-

bage, carrot, sugar beet, etc.) and fruits with symptoms of rot processes and from waste water and water from open water reservoirs. Investigated samples were centrifuged and handled by chloroform. The objects of research became phage, isolated from samples of carrot with symptoms of rot processes.

**Spot-titer assay.**The samples or serial dilutions of samples were applied dropwise on the plates with seeded bacterial culture. Following 20 minutes they were kept at room temperature in order to samples diffused into agar medium. Then plates were overturned and incubated in a thermostat at $37^0$C for 12 hours. After that the plates were analyzed for the presence of phages. Results were recorded as the reciprocal of the highest dilution at which clearing the lawn was evident.

**Double agar layer method.**0.2 ml of overnight bacterial culture ($10^8$ c.f.u./ml) was put together with 2.5 ml of 0.7% agar (the temperature of agar was 46-$49^0$C). Then 1 ml of the studied sample was added. The resulting mixture was accumulated on the bottom layer of 1,4% agar [1]. According to the results of spot-test, the concentration of phage particles in a sample of carrot was very high, so we diluted the phage lyzate to the $10^{th}$ degree in order to get separated plaques. After exposure within 15 minutes at the room temperature, plates were inverted and incubated at $37^0$C for 12 hours. After incubation all resulting plaques were counted. Separate phage plaques were then picked. Isolated bacteriophages were purified by serial propagation of single plaques and amplified.

**Electron microscopy.**Morphology of virions was investigated using the electron microscope. Formvar films placed on 400-mesh copper grids were dipped into sample for 2 min and contrasted in 2% uranyl acetate. The preparations were dried and viewed under the electron microscope at an instrumental magnification of 20,000.

**SDS-PAGE.**Structural proteins of phage isolates were analyzed by SDS-polyacrylamide gel electrophoresis [4]. Phage particles purified by ultracentrifugation were mixed with the sample buffer and then heated in a boiling water bath for 3 min, followed by separating the proteins in the gel (12%). Protein bands were visualized by staining the gels with Coomassie brilliant blue.

**Investigation of bacteriophages influence on the expression of pathogenic properties of the bacteria.**

In laboratory conditions the infectious process was modeled with the defeat of onions by investigated bacteria *Serratia marcescens*. Onion bulbs were disinfected with a solution of potassium hypermanganate for 20 minutes. Onion bulbs were divided on scales in sterile conditions and placed in plates on sterilized filter paper discs. Then 10mkl of phage mixture (titer $10^{10}$) and bacterial suspension (concentration of cells $10^8$ c.f.u./ml) were applied on the scales. For controlling the initiation of pathogenic process with bacteria a drop of bacterial suspension was applied on the scales of onion, placed in other plate. Onion scales with deposited drop of physiological solution served as a control of the experiment. Plates with the studied materials were incubated in a thermostat at $28^0$C during the week. For statistical significance of data each experiment was conducted in three repetitions.

**Results and discussion.**Plant material and water from two regions of Ukraine were screened for bacteriophages active against *Serratia marcescens* using a spot-test and double-agar technique. The agents capable to case the lysis of investigated bacteria were detected in carrot, sugar beet, onion, apples, water from career (tabl. 1).

***Table* 1. The research results of presence of *S. marcescens* phages in samples of different origins**

| Sample | Thepresenceof lysis zones | Thepresenceplaques after re-seeding |
|---|---|---|
| Carrot *Vitaminna* | + | + |
| Potato*Vesta* | - | - |
| Cabbage Caporal | - | - |
| Sugar beet Cylindra | + | - |
| Sugar beet Bordo | - | - |
| Pepper Diabolo | - | - |
| Onion Snow Ball | + | - |
| Onion Buran | - | - |
| Apple Gloster | + | - |
| Apple Goldstar | + | - |
| RaddishMantagong | - | - |
| Cauliflower Moravia | - | - |
| Waterfromthesettlersewage | - | - |
| Water from well | - | - |
| Water from lake | - | - |
| Water from river | - | - |
| Water from spring | - | - |
| Waterfromcareer | + | - |
| Water from pond | - | - |
| Mule | - | - |
| Beetpulp | - | - |
| Silos | - | - |

Then lytic spots were picked from agar and re-seeded on relevant strain of bacteria *Serratia marcescens* by the double agar layer procedure. It was observed that the majority of samples did not form negative colonies, with the exception of sample of carrot with symptoms of rot processes. The further work was carried out with this sample. After seeding the described sample on the *S. marcescens* lawn separate plaques with a clear edge 0,2-0,4 cm in diameter were received (fig. 1).

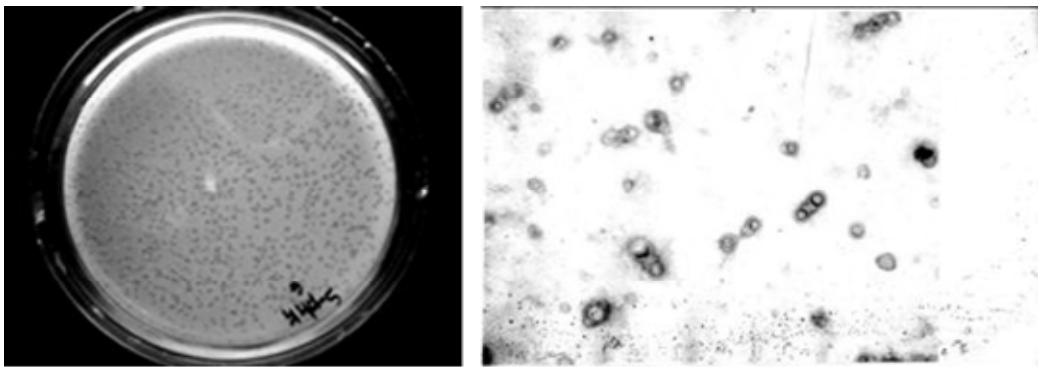

**Fig.1. Phage plaques and electron micrographs of *Serratia marcescens* phages**

The presence of phages in carrot samples was conclusively confirmed by TEM examination of concentrated phage filtrates (Fig. 1). Electron microscopy has revealed spherical phage particles without tails (fig. 1). According to the literary data it have not been identified viral particles of similar morphology for *S. marcescens* earlier. Therefore that is a problem of establishing their taxonomic affiliation. Newly isolated bacteriophage of *Serratia marcescens* was denoted as phage Smd1 and accumulated in high titer ($10^{12}$).

Six major protein bands (86,6 kDa, 45 kDa, 41,9 kDa, 35 kDa, 30,6 kDa, 21,4 kDa,) were identified after SDS-polyacrylamide gel electrophoresis of phage Smd1 proteins (Fig. 2).

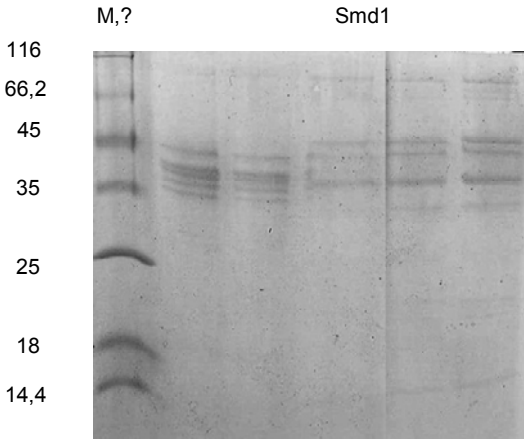


**Fig.2. Protein gel profiles for purified virions of bacteriophage Smd1**

As part of the experiments an impact of phage Smd1 on the manifestation of pathogenic properties of the investigated phytopathogenic strain of *S.marcescens* was modeled. Retrieved results showed that on control onion scales with deposited physiological solution and the scales with deposited phage and bacteria the processes of deterioration and rotting were not observed. On control scales with deposited overnight bacteria culture the gradual development of rotting processes was observed (fig. 3)..

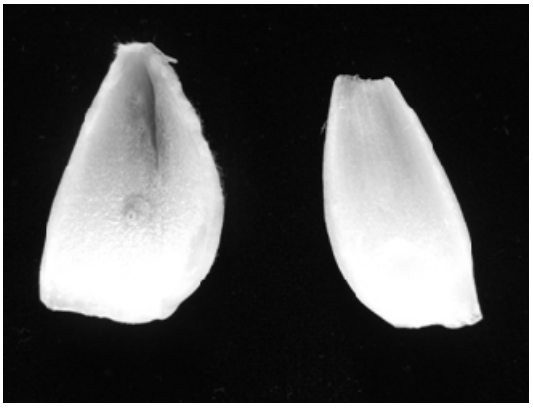


**Fig. 4. An Impact of phage Smd1 on manifestation of pathogenic properties of bacteria *Serratia marcescens*:**
A – onion scales with deposited suspension of bacterial culture;
B – onion scales with deposited suspension of bacteria and phage Smd1

**Conclusion.**Bacteriophage Smd1 was isolated from carrot and characterized. This phage is spesific to bacteria *Serratia marcescens* IMBG291 that case an onion diseases. Newly isolated phage morphologically and for it's proteins profiles differ from another known for today phages of *Serratia marcescens.* Taxonomy affiliation of bacteriophageSmd1 should be established. This phage can be useful for rapid diagnosis of *Serratia marcescens.* Obviously, the researched phage suppressed the growth of bacteriain a modelsystem. This leads us to suggestthat newly isolated phage can be used effectively as part of integrated onion disease management strategies.

1. Adams M. I. Bacteriophages. – New York, 1959. 2. Goodridge L.D. Bacteriophage biocontrol of plant pathogens:fact or fiction// TRENDS in Biotechnology. – 2004. – V.22, No.8. – P. 384-385. 3. Jones J.B., Jackson L.E., Balogh B.et.al. Bacteriophages for Plant Disease Control // Annu. Rev. Phytopathol. – 2007. – № 45. – P. 245–262. 4. Laemmli U. Cleavage of structural proteins during assembly of the head of bacteriophage T4 // Nature. – 1970. – Vol. 227, №15. – P. 608-685. 5.Ovcharenko L.P., Voznyuk T.M., Zaetz I.E. et.al. A mobile genetic element in Serratia marcescens, a causative agent of onion disease // Biopolymers and Cell. – 2010. – Vol. 26, № 4. – P. 279-285.

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# PECULIARITIES OF PHYLOGENY AND EXPRESSION OF TAS3-LIKE SMALL RNA PRECURSOR GENES IN PLANTS BELONGING TO FAMILY *ASTERACEAE*: EXPERIMENTAL APPROACH AND BIOINFORMATICS ANALYSIS

***Используя разработанную ранее нами методику для детекции и клонирования генов малых транс-действующих интерферирующих РНК (TAS3 генов), мы показали, что во взрослых растениях трех видов родов Curio и Senecio происходит экспрессия только одного типа TAS3 генов. Более того, анализ баз данных последовательностей кДНК у других родов в семействе Asteraceae также показал преимущественную экспрессию только одного TAS гена, названного нами TAS3-Sen1 и проявляющего значительную межвидовую гомологию в пределах этого семейства. Более того, при транскрипции гена TAS3-Sen1 у растений рода Senecio образуется не единственная РНК (первичный транскрипт ta-siRNA), а набор РНК за счет преждевременной терминации транскрипции внутри гена TAS3-Sen1. Обнаружение нами в составе наиболее протяженногоTAS3-Sen1 РНК-транскрипта повторяющейся последовательности (GA)n, учитывая данные литературных источников, позволяют предположить ее участие в синтезе недостроенных транскриптов.***

***In this paper, we have studied the presence of tasiARF RNA genes in the representatives of genus Seneciohaving either unifacial or true bifacial leaves. It was found that both types of species encode such RNA precursors principally similar to those found in Arabidopsis. We used primers mimicking miR390 and oligo (dT)-based oligonucleotides. One of the TAS3 species (TAS3-Sen1) in Senecio representatives was actively transcribed and distributed among many Asteracea plants and found to be similar to AtTAS3a. The data obtained may contribute to understanding of expression regulation of Senecio TAS3-like genes and the potential involvement of these RNAs in development of plant organ abaxial-adaxial symmetry.***

**Introduction.** In eukaryotes, small RNAs (sRNAs) exert transcriptional and post transcriptional control of genome expression to modulate development and responses to environmental stimuli [1–3]. Generally, sRNAs are inhibitors of gene expression that guide bound nuclease proteins to target nucleic acids via base-pairing interactions [3]. In the model plant *Arabidopsis thaliana*, sRNA biogenesis is catalyzed by homologues of the ribonuclease Dicer-like, DCL, that use double-stranded (ds) RNA as a substrate [4]. In plants, sRNAs can be broadly classified as microRNAs (miRNAs) and small interfering RNAs (siRNAs) [2, 3]. The genes encoding miRNA are transcribed by RNA polymerase II into primary transcripts containing a local stem-loop structure that provides the substrate for DCL1 cleavage into mature miRNAs of 21–22 nucleotides [1, 3]. miRNAs have a big impact as they negatively target cognate mRNAs for destruction or translational arrest [3]. Vascular plants including angiosperms (eudicots and monocots), gymnosperms and pteridophytes contain a repertory of miRNAs that are evolutionary conserved and control a large set of fundamental processes in plant development and homeostasis [5].

The various classes of plant 21- to 24-nt siRNAs derive from long dsRNA precursors that are processed by DCL2, DCL3 and DCL4 [1-3, 5]. The biosynthesis of these long dsRNA precursors usually entails the activity of one of several cell RNA-dependent RNA polymerases (RDRs) as well as plant viral RDRs [5]. In addition to virus-specific replicative-related double-stranded RNAs, sources of siRNAs include repetitive sequences, transposons, centromeres, convergent mRNA transcripts and other natural sense-antisense pairs, duplexes involving pseudogene-derived antisense transcripts and the sense mRNA from their cognate genes, hairpin RNAs as well as trans-acting siRNA (ta-siRNA)-generating transcripts (TAS) [1-6]. Endogenous siRNAs inactivate homologous sequences by a variety of mechanisms that include canonical post-transcriptional gene silencing as well as chromatin-dependent gene silencing [7].

The species of ta-siRNA were originally discovered in *Arabidopsis* [8]. Four gene families have been identified in *Arabidopsis* that each produce a number of ta-siRNAs: TAS1, TAS2, TAS3 and TAS4. Within the TAS1 family, TAS1a, TAS1b and TAS1c are very similar in sequence and all produce the siR255 ta-siRNA among others [6, 8]. One of the most important ta-siRNAs is the trans-acting short-interfering RNA-auxin response factor (tasiR-ARF). Auxins are a class of signaling molecules that play a central role in plant development. TasiR-ARF targets the mRNA of three Auxin Response Factor (ARF) genes (ARF2, ARF3/ETT and ARF4) for degradation [8]. TasiR-ARF is derived from the TAS3 gene. While *Arabidopsis* contains several ta-siRNAs not found in other plants, tasiR-ARF is highly conserved in all of these systems including mosses [6, 9]. This indicates that, like miRNAs, ta-siRNAs (and tasiR-ARF in particular) have been used to regulate gene expression in plants since before the separation between the seed plant and moss lineages [6, 8].

Each higher plant TAS gene so far identified produces a non-protein coding transcript that contains multiple ta-siRNAs within it. Each of these ta-siRNAs are lined up one after the other in both sense and anti-sense orientations. After transcription of the TAS gene, specific miRNAs pair with certain members of the Argonaute (AGO) protein family and bind to the single stranded RNA (ssRNA) at miRNA recognition sites [6, 10]. This specifies site specific cleavage of the primary TAS gene transcript at the beginning of the first ta-siRNA in the series and sets the phase for future processing [2, 6-8]. TAS3 gene transcript contain two miRNA recognition sites. Nevertheless only second (3'-terminal) site is cleaved AGO7 and downstream

miRNA-guided cleavage of the transcript is usually required for phased ta-siRNA biogenesis [2, 5, 6, 10]. At this point, Suppressor of Gene Silencing 3 (SGS3) protects the ssRNA from degradation. Next, RNA-dependent Polymerase 6 (RDR6) produces a complementary strand, turning the transcript into double stranded RNA (dsRNA) until non-cleaved site of TAS3 transcript bound to AGO-miRNA comples [6, 10]. Dicer-like Protein 4 (DCL4) then cleaves the dsRNA in 21-nt increments to generate mature ta-siRNAs [4, 6, 10].

The TAS3 gene-mediated ta-siRNA pathway is a plant-specific miR390-dependent phenomenon conserved in all higher plants and in primitive land plants such as *Physcomitrella* and other mosses [6, 9]. In *Arabidopsis*, miR390-guided cleavage occurs only at the 3′ target site of the TAS3 precursor where canonical targeting rules include perfect complementarity between miRNA nucleotides 2–13 (from the 5' end) and the mRNA target [6] (Fig. 1). The 5′ target site is resistant to cleavage but is important for processing of the tasiRNA precursor transcript. This characteristic feature is attributed to mismatches at nucleotides 9–12 from the 5′ end of the miRNA (Fig. 1). These mismatches are highly conserved in the higher plant TAS3-primary transcripts [6, 8].

In this paper, we have studied the presence of tasiARF RNA genes in the representatives of genus *Senecio*having either unifacial or true bifacial leaves. It was found that both types of species encode such RNA precursors principally similar to those found in Arabidopsis. We used primers mimicking miR390 (Krasnikova et al., 2009) and oligo (dT)-based oligonucleotides. One of the TAS3 species (TAS3-Sen1) in Senecio representatives was actively transcribed and distributed among many *Asteracea* plants and found to be similar to AtTAS3a. The data obtained may contribute to understanding of expression regulation of *Senecio* TAS3-like genes and the potential involvement of these RNAs in development of plant organ abaxial-adaxial symmetry.

**Materials and Methods**. *Plant material*. Specimens for *Senecio talinoides, Curio repens* and *Curio articulatus* were taken from collections of the N.V. Cycin Main Botanic Garden of the Russian Academy of Sciences. *Analysis of Nucleic Acids.*Genomic plant DNA was isolated from 200 mg of plant material by DNA extraction kit (Macherey-Nagel) according to the protocol of the manufacturer. TAS3 genes were amplified and sequences as described in [11]. Total RNA was isolated from *Nicotiana benthamiana* leaves with the Trizol reagent according to the manufacturer's instructions (Invitrogen). Digestion of any contaminating DNA was achieved by treatment of samples with RQI RNase-free DNase (Promega). Reverse transcription was performed with 1 *μ*g of total RNA and oligo (dT)-primer t20-xho (attctcgaggccgaggcggccgacatgttttttttttttttttttttttttttv) using the RT system (Invitrogen) according to the protocol of the manufacturer. Primers for dicotyledonous plants were: forward primer: TAS-P (5'-GGTGCTATCCTATCTGAGCTT-3')and mixture of reverse primers TAS-Mcaa (5'-AGCTCAGGAGGGATAGCAA-3')and TAS-Maca (5'-AGCTCAGGAGGGATAGACA-3'). For PCR, 25–35 cycles were used for amplification with a melting temperature of 95°C, an annealing temperature of 58°C and an extending temperature of 72°C, each for 30 seconds, followed by a final extension at 72°C for 3 min. PCR products were separated by electrophoresis of samples in 1.5% agarose gel and purified using the GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences). For cloning, the PCR-amplified DNA bands isolated from gel were ligated into pGEM-T (Promega). Cloned products were used as templates in sequencing reactions with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). DNA sequences were deposited at the NCBI data bank, the accession numbers are JN692262, JN692261, JN692260 and JN692259. The TAS3 cDNA sequences were cut out of the vectors pGEM-T by digestion with HindIII and ligated into HindIII-digested binary vector pLH7000 containing the flanking 35S promoter and transcriptional terminator and provided by Dr L. Hausmann (Federal Centre for Breeding Research on Cultivated Plants, Germany). Agroinfiltration of *Nicotiana benthamiana* plants was carried out as described [12]. RNA was isolated 4 days after agroinfiltration of plant leaves (see above).

**Results and Discussion.***Identification and molecular phylogeny of the most abundant TAS3-like species in representatives of family Asteraceae.*Leaves of angiosperms exhibit considerable morphological diversity and thus represent an attractive subject for developmental molecular studies [13]. The diverse leaf forms in angiosperms can be categorized as bifacial or unifacial. Bifacial leaves, such as those of Arabidopsis thaliana, are the more typical form of leaves that differentiate adaxial-abaxial (upperlower) polarity. The establishment of adaxial-abaxial polarity in bifacial leaves is regulated by overlapping and antagonistic genetic interactions involving several distinct transcription factors and small regulatory RNAs [14]. Unifacial leaves, which are characterized by an abaxialized leaf blade, have repeatedly evolved in a number of divergent monocot species [15]. Transcription factors *ARF3* and *ARF4* are required for specifying leaf abaxial identity [14]. On the other hand, *ARF3*/*4* transcripts are targets of a *TAS3*-derived *trans*-acting short interfering RNA, tasiR-ARF, which guides the cleavage of *ARF3/4* mRNAs (see above). The *Arabidopsis* TAS3 is expressed on the adaxial side of early leaf primordia, which possibly regulates the expression of ARF3 and ARF4 in the adaxial domain and determines the dorso-ventral leaf polarity [16]. Genetic analyses in maize and rice have also demonstrated a primary role for ta-siARF during dorsiventral leaf patterning [17, 18]. To further study the role of TAS3 ta-siRNA in development of leaf polarity in dicot plants, we started the studies of some representatives of family *Asteraceae* where some succulent plants of Senecioneae are characterized by an abaxialized unifacial leaf blade [19].

The *Asteraceae* is the second largest family of flowering plants with at least 1100 genera and over 20,000 species included. The *Asteraceae* includes shrubs and a few trees. Senecioneae is the largest tribe of *Asteraceae*, comprised of ca. 150 genera and 3,000 species. Approximately one-third of its species are placed in *Senecio*, making it one of the largest genera of flowering plants. Despite considerable efforts to classify and understand the striking morphological diversity in Senecioneae, little is known about its intergeneric relationships. This lack of phylogenetic understanding is predominantly caused by conflicting clues from morphological characters, the large size of the tribe, and the absence of a good delimitation of *Senecio*. Phylogenetic analyses of nuclear ribosomal ITS and plastid DNA sequence data were used to produce a hypothesis of evolutionary relationships in Senecioneae [20].

Previously, we described the new method for identification of plant ta-siRNA precursor genes based on PCR with oligodeoxyribonucleotide primers mimicking miR390. The method was found to be efficient for dicotyledonous plants, cycads, conifers and mosses [9, 11]. In this study, PCR-based approach was used as a tool to probe genomic DNA samples derived from three species of Senecioneae: *Senecio talinoides* with abaxialized unifacial leaves, *Curio repens* with pseudo-unifacial leaves and *Curio articulatus* with bifacial leaves [19]. PCR amplification of chromosomal DNA from these plants resulted in synthesis of one major band of 270 bp (data not shown). Cloning and sequencing

of the obtained DNA bands revealed that the amplified sequences are closely related (more than 90% identity) (data not shown) contained putative ta-siARF site composed of two tandem copies of ARF-specific ta-siRNAs and located between miR390 target sites corresponding to PCR primers (Fig. 1). This TAS3-like gene was named as TAS3-Sen1 (GenBank accession number – JN692259).

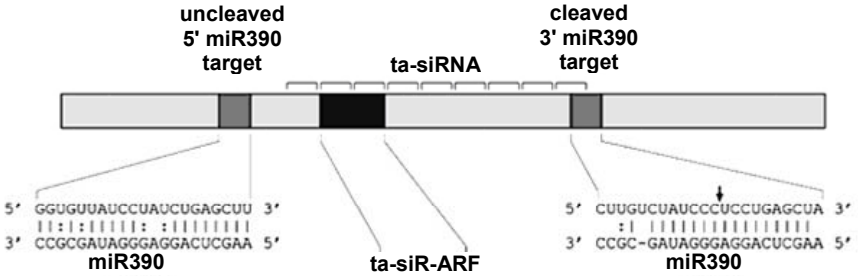


**Figure 1. Organization of TAS3 tasiRNA precursors. The miR390-guided cleavage site is indicated by the arrow. The tasiRNA region is indicated by brackets.**

Bioinformatic analysis of the putative TAS3-like sequences from *Senecio talinoides, Curio repens* and *Curio articulatus* using NCBI Blast revealed closely related sequences in other representatives of the family *Asteraceae* (data not shown and Fig. 2). Using available sequence databases, more distant TAS3-like sequences were identified at least in 2 families belonging to subclass Rosids and 11 families of flowering plants belonging to subclass Rosids, for which complete sequences of TAS3 cDNAs were revealed (see Materials and Methods; data not shown and Fig. 2). The phylogenetic tree based on comparisons of TAS3-like sequences demonstrated that all TAS3-Sen1 sequences of *Asteraceae* form a monophyletic group (Fig. 2). On the other hand, the closest branching lineage in eudicots was represented by plants from subclass Asterids, namely,orders Lamiales (*Antirrhinum majus*) and Solanales (*Nicotiana tabacum* and *Solanum tuberosum*) (Fig. 2). These observations are in agreement with the branching order of flowering plant evolution trees published previously [21].

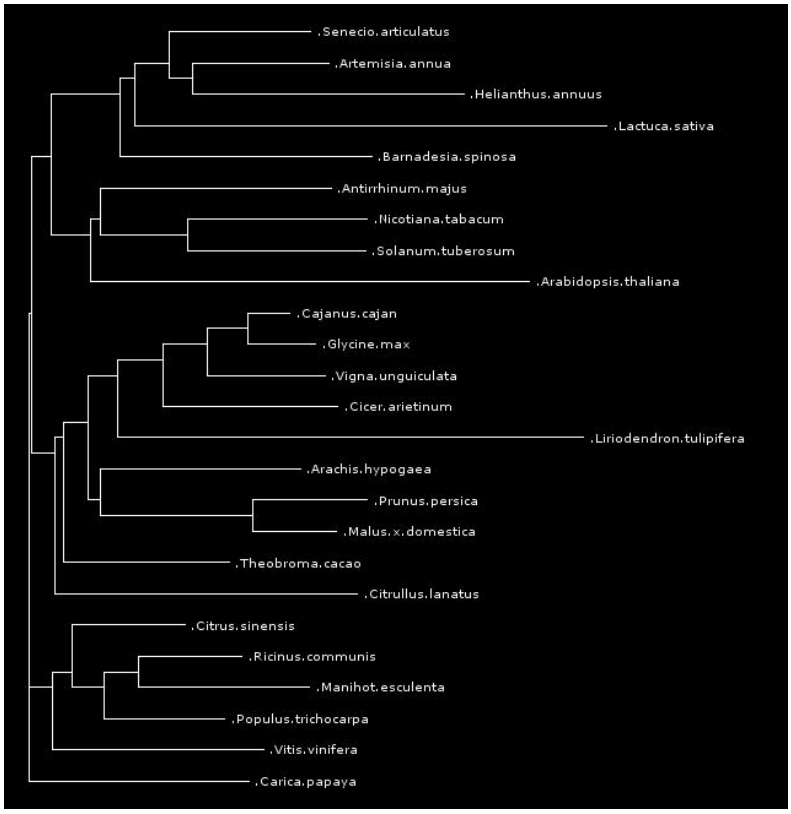


**Figure. 2. The minimal evolution phylogenetic tree based on analysis of the aligned TAS3 genes from some flowering plants. This tree was generated according MAFFT6 program (http://mafft.cbrc.jp/alignment/server)**

The TAS3-Sen1 family like other other TAS3 loci is characterized by the dual miR390 binding sites, which are functionally required by TAS3 mRNA to define the phasing register for tasiRNA production [6]. We conducted an exhaustive Blast search using TAS3-Sen1 genes as queries to find additional putative Senecio TAS3 genes in the NCBI databases. The *Senecio madagascariensis* cDNA contig (GenBank accession number SRR006592) includes the tasiARF region and flanking 5'-terminal miR390 complementary site. However, this cDNA contig was shorter than

the full-length TAS3 mRNA precursor and 3'-terminal miR390 site could not be mapped. Nevertheless, we used sequence of contig SRR006592 to reconstruct the authentic sequence (not derived from PCR primer mimicking miR390) of 5'-terminal miR390 complementary site (Fig. 3A). Therefore, the presence of miR390 complementary sites in the TAS3-Sen1 loci supported the idea that they were indeed tasiRNA genes, and suggested that miR390 set the phasing register of tasiARFs (Fig. 3A). Indeed, ta-siARF sequences in TAS3-Sen1 coaligned with the phases D7(+) and D8(+) defined by the 3′ miR390 processing site in their putative precursors as observed in angiosperms (Figs. 1 and 3A). [6, 22].TAS3-Sen1 loci in S.articulatus and S.madagascariensis both had identical region including two closely related 21-nt sequences adjacent to one another of which one was identical to Arabidopsis tasiARF at phase 5′D8(+) and the other contained three mismatches with respect to the Arabidopsis tasiARF at position 5′D7(+) (Fig. 3B). To verify whether the interaction between Senecio miR390 and the TAS3-Sen1 precursor is similar to what was observed in *Arabidopsis*, sequence comparison was performed to predict cleavage events at the 5′ and 3′ target sites on the TAS3 precursor. Canonical targeting rule including nearly perfect complementarity between miR390 nucleotides 2–13 (from the 5' end) and the TAS3 RNA 3'-terminal miR390 target was confirmed (Fig. 3). These results are also consistent with the previous suggestion that miR390 is unable to guide for a cleavage because of mismatches between 9 nt and 11 nt from the 5′ end of the miR390 [6, 22] (Fig. 3).

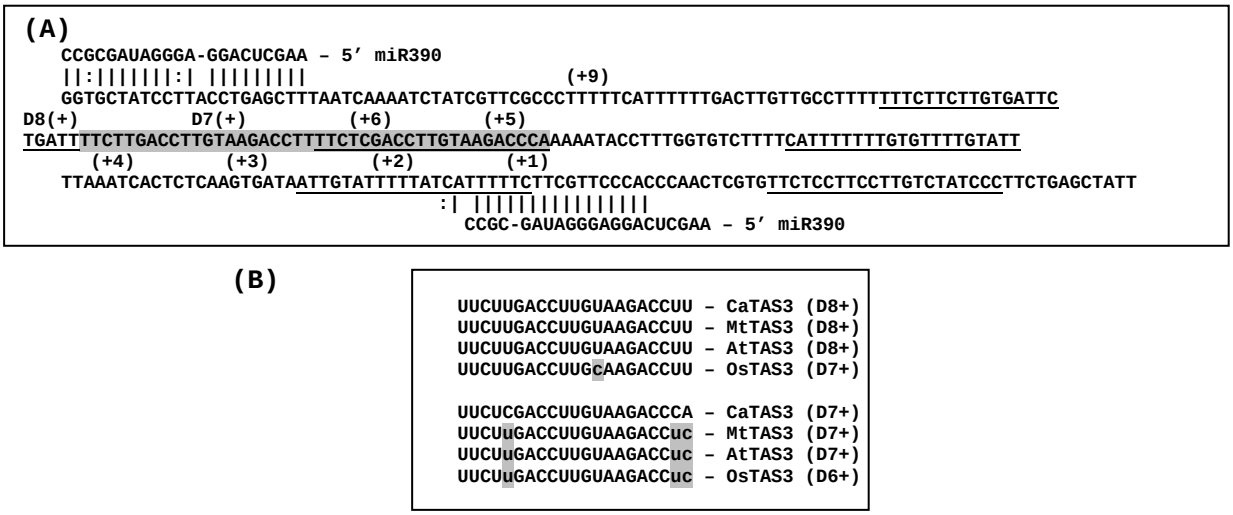


**Figure 3. (A) Nucleotide sequence of TAS3-Sen1 locus. The 5′ and 3′ miR390 target sites are shown as alignments. Predicted Dicer processing sites are shown by underlining. TAS3-Sen1 siRNAs that are complementary to the auxin response factors are indicated by shading (+D7TAS3 and +D8TAS3).**
**(B) Sequence alignment of the TAS3-Sen1 and TAS3 ta-siARF RNAs from Arabidopsis, rice and *M. truncatula***

*Cloning and sequencing of the TAS3-Sen1 cDNA species suggests a new strategy for the involvement of non-cleavable miR390 target site in control of ta-siARF formation.* Processing and polyadenylation of plant TAS3 gene precursors has not been studied in detail excepting splicing of Arabidopsis thaliana gene TAS3a (AT3G17185) [23]. We have studied the polyadenylation sites of TAS3-Sen1 genes in three distinct Senecio species by sequence analysis of multiple cDNA clones. Figure 4 shows the nucleotide sequence of the 3' tail regions following ta-siARF tandem region of TAS3-Sen1 gene in three plant species and the polyadenylationsites of the analyzed cDNA clones. The cDNA clones for the genes of *S. talinoides, C. repens and C. articulatus* fall into groups with differentpolyadenylation sites. The two putative full-length St. 41 and Ca. 990 cDNA clones analyzed also had different poly A sites. Since the poly A tails of all the cDNA clones analyzed do not occur in the regions corresponding to a long AT rich area in the genomic sequence we are confident that correct priming occurred with the oligo dT during the cDNA preparation, and that the different groups of cDNA clones represent true differences in the poly A addition site on the mRNA's. In the case of Ca.91, St.33 and St.33 species many cDNA clones have been revealed for each gene. Eight out of 12 of the cDNA clones for *C. repens* and 7 out of 13 clones for *C. articulatus* had the poly A tail added in this middle position. The sites where polyadenylation occurs in the mRNA are spread over quite large distances (Fig. 4 and data not shown). The two polyadenylation sites detected for *C. articulatus* are 216 nucleotides apart, those for *S. talinoides* are separated by 269 nucleotides; however the three sites were detected for *C.* repenst. A high degree of variability in the positionning of the poly A tails therefore occurs between these highly related genes in three plant species. One feature which is common to Cr.29 and Cr.31 species is that the nucleotide in the genomic sequence which corresponds to the first A in the poly A tails of the cDNA clones, is an A residue. Also illustrated in Figure 4 is sequence in St.41 which may represent the G/A cluster sequences positioned immediately upstream of the poly A addition site. The potential importance of homopurine-homopyrimidine sequences in biological processes is suggested by the ubiquity and non-random distribution of sequences such as (CT)n'(GA)n within the genomes of many organisms [24]. GA repeats have been found in promoter regions of many genes, at recombination hotspots and at replication origins [24]. Homopurine-homopyrimidine sequences could influence transcription through several possible mechanisms. It has been suggested that the tendency of these sequences to adopt alternative DNA conformations such as H-DNA could be used to absorb negative supercoils generated in the wake of RNA polymerase, facilitating DNA unwinding within the transcription bubble [24, 25]. Homopurin-homopyrimidine sequences could also influence chromatin structure by serving as binding sites for regulatory proteins or important chromatin assembly fac-

tors. This possibility has been particularly well studied in the case of the Drosophila hsp26 gene [25]. GAGA binding factor is believed to affect several steps in the process of transcription particularly causing RNA polymerase II pausing in many genes during transcription [25]. This phenomenon may be related to premature termination of gene transcription.

To test the possibility that homopurine-homopyrimidine sequences may influence premature termination of TAS3 gene transcription we inserted the clone St.41 into agrobacterial vector for transient expression. Epidermal cells of *N. benthamiana* leaves were agroinfiltrated with this construct where transcription was driven by 35S promoter. At 4 days post infiltration total RNA was isolated. Three out of 9 clones for transiently expressed *C. articulatus* TAS3-Sen1 had the poly A tail added in the middle position (similar to clone St.33 – Fig. 4). The rest of the clones were terminated at the position typical for cauliflower mosaic virus 35S terminator (data not shown). No clones analogous to St.41 (possessing G/A cluster upsteam of poly (A) were observed for infiltrated leaf areas (data not shown).

```
St.41 – TTCTTGACCTTGTAAGACCTTTTCTCGACCTTGTAAGACCCAAAAATACCTTTGGTGTCTTTTCATTTTTTTGTGTTTTGTATTTTAAATCACTCTCAAGTG
Ca.90 – ------------------------T-----------------------------------------C-----C-------G------------T-------
Ca.91 – ------------------------T-----------------------------------------C-----C-------G------------T-------
St.33 – ----------------------------------------------------------------------------------------------------
Cr.28 – ------------------------T-----------------------------------------------------G--------------------
Cr.29 – ------------------------T-----------------------------------------------------G----- – poly(A)
Cr.31 – ------------------------T---------- poly(A)


ATAATTGTATTTTTATCATTTTTCTTCGTTCCCACCCAACTCGTGTTCTCCTTCCTTGTCTATCCCTTCTGAGCTATTTCCTCTCACAATCAACTAGTCAAGGGTTATGT
-----------------G-----------------------A---------------------------------------T-G----------------##--
----------------G----------- – poly(A)
---------------------------- – poly(A)
---------------------------- – poly(A)

GTTTGTTCCAACTCGGAATTAATTAGTTGGTAGAAACAAGCCCTAATATCTCTTTGTTAGTAAACAAAATGTAACCTTATTTCTTGGTCTCCTTCTACAAAACAAGAGCC
----T------------------G-----------------------A---##############################----------G----------T------

AAAACCTTTCGTCCGTCATCTTTAAAAATGCTCCATATATGATGGTATATTAGAGAGAGAGAGAGAGAGAGAGAGAG – poly(A)
-----------------------------------AG-T---ACGACG---TAG – poly(A)
```

**Figure 4. Nucleotide sequences of full-length and premature terminated transcripts of TAS3-Sen1 genes from S. talinoides (St.), C. repens (Cr.) and C. articulatus (Ca.). TAS3 ta-siARF region is shaded. Deleted residues are shown as (#)**

It was shown recently that alternative polyadenylation would generate two transcripts from the *Arabidopsis* TAS3a locus (At3g17185), only one of which contains both miR390 binding sites flanking ta-siARF region and would be subject to miR390-mediated processing [26, 27]. Such phenomenon allows for more layers of regulation linked to miRNA and ta-siRNA. For example, the non-protein coding gene IPS1 (INDUCED BY PHOSPHATE STARVATION 1) from Arabidopsis thaliana contains a motif with sequence complementarity to the phosphate (Pi) starvation-induced miRNA miR-399, but the pairing is interrupted by a mismatched loop at the expected miRNA cleavage site [28]. As a result IPS1 RNA is not cleaved but instead sequesters miR-399. Thus, IPS1 overexpression results in increased accumulation of the miR-399 target PHO2 mRNA and, concomitantly, in reduced shoot Pi content. Moreover it was found that over-expression of several additional miRNA decoys with non-cleavable miRNA sites result in a certain degree of specific inactivation of individual members of different miRNA families [29]. In principle, the application of alternative transcripts of Senecio TAS3 genes as decoys for the modulation of miR390 activity could be extended to regulate synthesis of ta-siARF RNAs in different Senecio species by permitting varying degrees of miR390 inactivation by their own functional TAS3 target genes.

1. Chen X. Small RNAs – secrets and surprises of the genome // Plant Journal. – 2010. – Vol. 61, № 6. – P. 941–958. 2. Axtell M. J., Westholm J. O., Lai, E. C. Vive la différence: biogenesis and evolution of microRNAs in plants and animals // Genome Biology. – 2011. – Vol. 12, № 4. – P. 221-234. 3. Xie Z., Khanna K., Ruan S. Expression of microRNAs and its regulation in plants // Semin. Cell. Dev. Biol. – 2010. – Vol. 21, №8. – P. 790-797. 4. Liu Q., Feng Y., Zhu Z. Dicer-like (DCL) proteins in plants // Funct. Integr.Genomics. – 2009. – Vol. 9, №3. – P. 277-286. 5. Xie Z., Qi X. Diverse small RNA-directed silencing pathways in plants // Biochimica et Biophysica Acta. – 2008. – Vol. 1779, №2. – P. 720–724. 6. Allen E., Howell M. D. miRNAs in the biogenesis of trans-acting siRNAs in higher plants // Semin. Cell. Dev. Biol. – 2010. – Vol. 21, №8. – P. 798-804. 7. Chan S. W-L. Inputs and outputs for chromatin-targeted RNAi // Trends in Plant Science. – 2008. – Vol. 13, №7. – P. 383-389. 8. Vaucheret H. MicroRNA-dependent trans-acting siRNA production // Science STKE. – 2005. – Vol. 3. – P. 43-48. 9. Krasnikova M. S., Milyutina I. A., Bobrova V. K., Troitsky A. V., Solovyev A. G., Morozov S. Y. Molecular Diversity of miR390-Guided Transacting siRNA Precursor Genes in Lower Land Plants: Experimental Approach and Bioinformatics Analysis // Sequencing. – 2011. – vol. 2011, Article ID 703683, doi:10.1155/2011/703683. 10. Takeda A., Iwasaki S., Watanabe T. The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins // Plant Cell Physiology. – 2008. – Vol.49, №4. – P. 493-500. 11. Krasnikova M. S., Milyutina I. A., Bobrova V. K., Ozerova L. V., Troitsky A. V., Solovyev A. G., Morozov S. Y. Novel miR390-dependent transacting siRNA Precursors in plants revealed by a PCR-based experimental approach and database analysis // Journal of Biomedicine and Biotechnology. – 2009. – Vol. 2009, Article ID 952304. 12. Yelina N. E, Erokhina T. N. Lukhovitskaya N. I. Localization of Poa semilatent virus cysteine-rich protein in peroxisomes is dispensable for its ability to suppress RNA silencing // J Gen Virol. – 2005. – Vol. 86. – P. 479–489. 13. Piazza P., Jasinski S., Tsiantis M. Evolution of leaf developmental mechanisms // New Phytol. – 2005. – Vol. 167, №3. – P. 693-710. 14. Husbands A.Y., Chitwood D. H., Plavskin Y., Timmermans M. C. Signals and prepatterns: new insights into organ polarity in plants // Genes Dev. – 2009. – Vol. 23, №17. – P. 1986-1997. 15. Yamaguchi T, Yano S, Tsukaya H. Genetic framework for flattened leaf blade formation in unifacial leaves of Juncus prismatocarpus // Plant Cell. – 2010. – Vol. 22, №7. – P. 2141-2155. 16. Garcia D., Collier S. A., Byrne M. E., Martienssen R. A. Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway //Current Biology. – 2006. – Vol. 16. – P. 933–938. 17. Nogueira F. T., Madi S., Chitwood D. H., Juarez M. T, Timmermans M. C. Two small regulatory RNAs establish opposing fates of a developmental axis //Genes & Development. -2007. – Vol. 21. – P. 750–755. 18. Douglas R. N., Wiley D., Sarkar A., Springer N., Timmermans M. C., Scanlon M. J. Ragged seedling2 encodes an ARGONAUTE7-like protein required for mediolateral expansion, but not dorsiventrality, of maize leaves // Plant Cell. – 2010. – Vol. 22, №5. – P. 1441-1451. 19. Ozerova L.V., Timonin A.C. On the evidence of subunifacial and unifacial leaves: Developmental studies in leaf-succulent Senecio L. species (Asteraceae) // Wulfenia. – 2009. – Vol. 16. – P. 61-77. 20. Bettin O., Cornejo C., Edwards P. J., Holderegger R. Phylogeography of the high alpine plant Senecio halleri (Asteraceae) in the European Alps: in situ glacial survival with postglacial stepwise dispersal into peripheral areas // Mol Ecol. – 2007. – Vol. 16, №12. – P. 2517-2524. 21. Duarte J. M., Wall P. K., Edger P.P., Landherr L.L., Ma H., Pires J.C., Leebens-Mack J., de Pamphilis, C.W. Identification of shared single copy nuclear genes in

Arabidopsis, Populus, Vitis and Oryza and their phylogenetic utility across various taxonomic levels // BMC Evol. Biol. – 2010. – Vol. 10. – P. 61-70. 22. Jagadeeswaran G., Zheng Y., Li Y. F., Shukla L.I., Matts J., Hoyt P., Macmil S. L., Wiley G. B., Roe B. A., Zhang W., Sunkar R. Cloning and characterization of small RNAs from Medicago truncatula reveals four novel legume-specific microRNA families // New Phytol. – 2009. – Vol. 184, №1. – P. 85-98. 23. Alexandrov, N.N., Troukhan, M.E., Brover, V.V., Tatarinova, T., Lu, Y.-P., Flavell,R.B., Feldmann,K.A. Features of Arabidopsis genes and genome discovered using full-length cDNAs // Plant Mol. Biol. – 2006. – Vol. 60, №1. – P. 69-85. 24. Quinn L., Teare J. M., Granok H., Swede M. J., Xu J., Elgin S.C.R. The capacity to form H-DNA cannot substitute for GAGA factor binding to a (CT)n'(GA)n regulatory site // Nucleic Acids Research. – 2003. – Vol. 31, No. 10. – P. 2483-2494. 25. Lee C., Li X., Hechmer A., Eisen M., Biggin M. D., Venters B. J., Jiang C., Li J., Pugh B. F., Gilmour D. S. NELF and GAGA Factor Are Linked to Promoter-Proximal Pausing at Many Genes in Drosophila // Mol Cell Biol. – 2008. – Vol. 28, №10. – P. 3290-3300. 26. Hunt A. G. RNA regulatory elements and polyadenylation in plants // Frontiers in Plant Science. Plant Genetics and Genomics. – 2012. – Vol. 2, article 109. 27. Wu X., Liu M., Downie B., Liang C., Ji G., Li Q.Q., Hunt A.G. Genome-wide landscape of polyadenylation in Arabidopsis provides evidence for extensive alternative polyadenylation // Proc Natl Acad Sci U S A. – 2011. – Vol. 108, №30. – P. 12533-12538. 28. Franco-Zorrilla J. M., Valli A., Todesco M., Mateos I., Puga M.I., Rubio-Somoza I., Leyva A., Weigel D., García J. A., Paz-Ares J. Target mimicry provides a new mechanism for regulation of microRNA activity // Nat Genet. – 2007. – Vol. 39, №8. – P. 1033-1037. 29. Ivashuta S., Banks I. R., Wiggins B. E., Zhang Y., Ziegler T. E., Roberts J. K., Heck G. R. Regulation of gene expression in plants through miRNA inactivation // PLoS One. – 2011. – Vo. 6, №6. – e21330.

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# GENETIC MONITORING OF CIRCULATING INFLUENZA VIRUSES IN UKRAINE DURING 2010-2011 INFLUENZA SEASON

***Результаты филогенетичского анализа украинских изолятов вируса гриппа эпидемического сезона 2010-2011 показали их схожесть с таковыми в мире. Анализ мутаций обнаружил важные аминокислотные замены в генах гемагглютинина украинских изолятов.***

***The phylogenetic analysis of Ukrainian influenza viruses during 2010-2011 epidemic season showed their similarity to flu isolates all around the world. Mutation finding indicated significant amino acid substitution in the hemagglutinin genes of Ukrainian viruses.***

Annual influenza epidemics in many countries, considerably in Northern Hemisphere, are caused by continuous variability of influenza viruses and extreme activity of their transmission. In fact, epidemic season 2010-2011 has lower intensity and les number of selected viruses from the previous 2009-2010 pandemic season.

Monitoring the genetic changes of influenza viruses appeared in our country not long ago, but has already proved its relevance and necessity. Such data can considerable speed up the adequate response to new or changed viruses. So, the work of all public health services in country was adjusted, after confirmating first selected pandemic A(H1N1) sw influenza isolate.

The aim of our study was to perform the genetic monitoring of influenza viruses isolated in Ukraine and to compare with viruses from other countries which circulated during the 2010-2011 epidemic season by constructing the phylogenetic trees.

**Materials and methods**. Nasopharyngeal swabs and autopsy materials collected from infected patients, were received during the epidemic from different regions of Ukraine. Typing of viruses was performed by real-time RT-PCR (reverse transcription polymerase latsyuhova reaction in real time). Strain definition was performed with using the diagnostic serum sent by Center for Disease Control and Prevention (CDC Atlanta, USA). For genetic analysis were used sequences of hemagglutinin gene of Ukrainian influenza viruses A(H1N1)sw, A(H3N2) and B. The GISAID web resource [1] was used for searching sequences essential for the analysis. The identification and comparison of obtained sequences was performed in BLAST (Basic Local Aligment Search Tool) [2]. Phylogenetic comparison and constructing the phylogenetic trees was performed in MEGA 5 program software [3] with using the Neighbor-Joining (combining nearest neighbors) [4] method. The aligning of sequences was performed with using ClustalW algorithm [5]. For the statistical evaluation of the results significance was used Bootstrap analysis with the number of replications 1000 [6].

**Results and discussion.** Influenza season 2010-2011 was the first after the pandemic season in Ukraine. The distinction of this season was early wide circulation of influenza B viruses (autumn 2010) and ongoing circulation of pandemic A(H1N1)sw influenza viruses (from the second half of January and until the beginning of April). And only two of A(H3N2) influenza viruses have been isolated. Genetic analysis was directed to hemagglutinin genes as those with high level of variability. More over the hemagglutinin genes are the primary target for neutralizing antibodies.

**Phylogenetic comparison of hemagglutinin (HA) genes of influenza Asw (H1N1) viruses**

The pandemic A (H1N1) sw influenza viruses continued to be isolated in the 2010-2011 epidemic season despite their appearence in early 2009 by triple gene recombination of human, swine and bird influenza viruses [7]. It is known from the literature that hemagglutinin of pandemic influenza viruses has classical swine flu virus derivation (like ones, caused the pandemic of 1918-1919), which distinguishes them from human or birds influenza viruses [8].

We began to research the hemagglutinin sequences of Ukrainian isolates from finding similar genetic sequences with using BLAST search too. So, we found viruses with high percentages of similarity for the hemagglutinin genes: isolate from a 36 years old man, who has ill after contact with a sick animal – A/Ohio/01/2007 (the similarity 93%), and swine influenza virus – A/swine/Illinois/1/1975 (similarity – 87%). Also in analysis were taken the reference strain of seasonal human influenza virus (H1N1) – A/New Caledonia/20/1999 with 7% similarity (Fig. 1.).

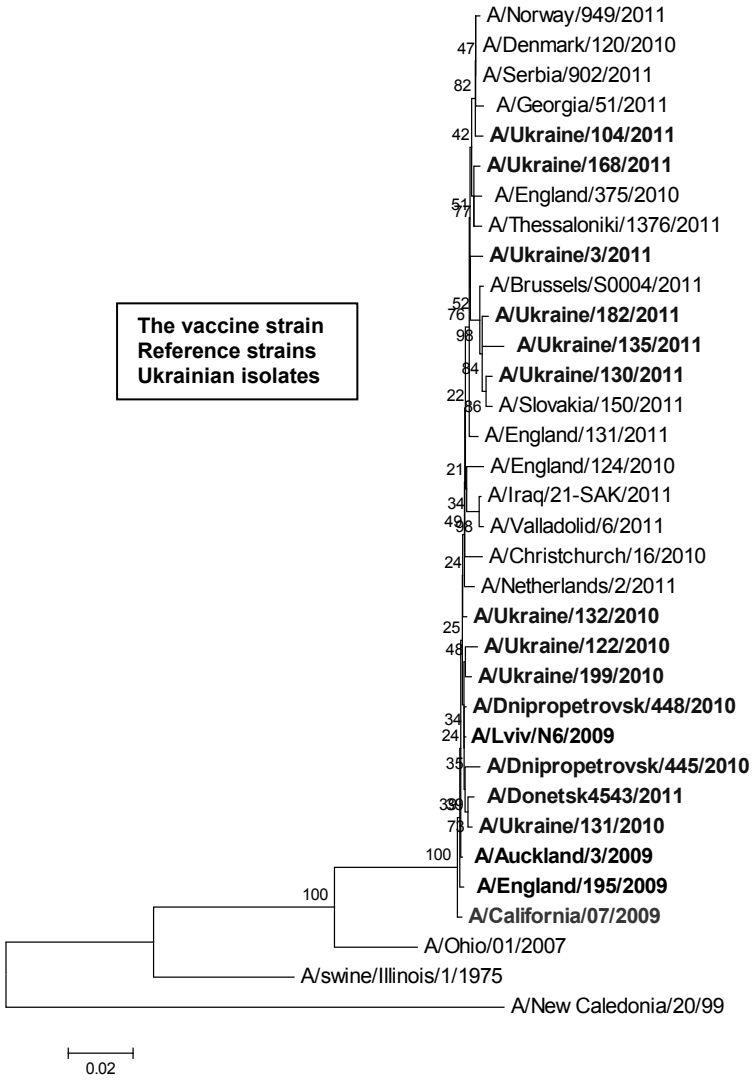


**Fig.1. Phylogenetic tree of hemagglutinin (HA) gene sequences of influenza A(H1N1)viruses constructed by NJ method with Kimura 2-parameter model (Bootstrap values are indicated)**

According to the results of Hemagglutinin inhibition (HI) assay our isolate A/Lviv/N6/2009 was selected as reference strain due to its low binding activity to ferret antisera comparing to the vaccine strain A/California/07/2009 (in titer of 160 against 1280).

Having completed all steps of analysis, we obtained phylogenetic tree where hemagglutinin genes of Ukrainian isolates comparably to viruses isolated in other countries, including vaccine and reference strains (Fig. 1.). We can see from the figure that viruses isolated in Ukraine in autumn 2010 placed at the bottom of phylogenetic tree near the reference strain A/Lviv/N6/2009. But isolates fron early 2011 had gained changes that determined them in another cluster. Also we have found one exception – A/Donetsk/4543/2011, which remained genetically similar to isolates from autumn 2010, although was isolated in 2011.

Also, for more detailed analysis of the hemagglutinin gene of pandemic influenza viruses, we constructed a phylogenetic tree, with only pandemic influenza viruses (Fig. 2.). More over, we find amino acid differences in sequences of hemagglutinin proteins of Ukrainian isolates in comparison with the vaccine and reference strains. Three isolates, A/Ukraine/130/2011, A/Ukraine/135/2011 and A/Ukraine/182/2011 formed a separate cluster with viruses isolated in Brussels and Slovakia according their differences. In hemagglutinin of these viruses were found following amino acid substitutions: S143G (glutamine changed serine at position 143) and A197T (threonine changed alanine at position 197).

In addition, isolate A/Ukraine/135/2011 showed polymorphism in the nucleotide sequence encoding the 155 amino acid residue of hemagglutinin protein. According to the World Influenza Centre (London) polymorphism or nucleotide substitution in the region, which encodes residues from 153 to 157 is often associated with low binding ability to antisera. It is interesting that the polymorphism in this region was not observed in clinical samples. It can be an artifact of reproduction in cell culture, especially for the MDCK-siat line, that ceased to use in study of influenza A(H1N1)sw viruses [9].

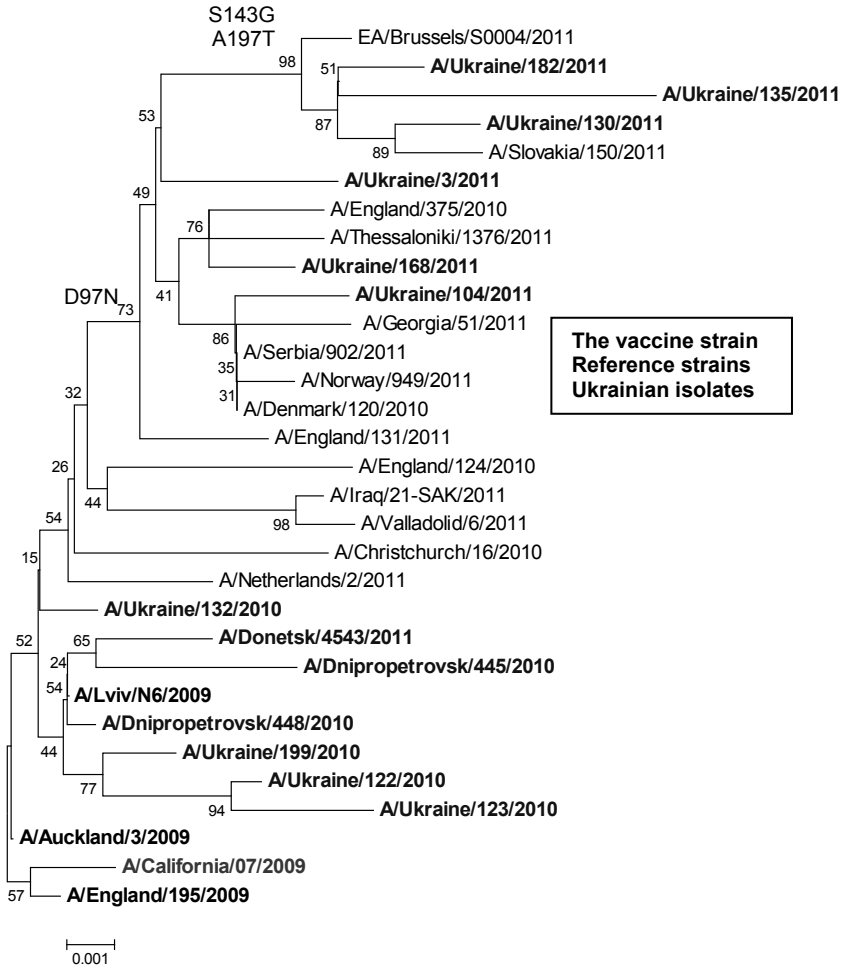


**Fig. 2. Phylogenetic tree of hemagglutinin (HA) gene sequences of pandemic influenza A(H1N1)sw viruses constructed by NJ method with Kimura 2-parameter model (Bootstrap values are indicated)**

All pandemic influenza viruses isolated in our country has preserved similarity to vaccine strain A/California/07/2009 near 98%, but showed minor changes in the hemagglutinin genes(Fig. 2.).

**Phylogenetic comparison of hemagglutinin (HA) genes of influenza B viruses**

All isolated and sequenced influenza viruses type B belonged to B/Victoria/2/87genetic branch that was dominating worldwide during this epidemic season (about 85%). As we can see from the phylogenetic tree (Fig. 3) all Ukrainian isolates (marked blue) were similar to the vaccine strain B/Brisbane/60/2008, except few mutations.

Amino acid substitutions in hemagglutinin of Ukrainian influenza B viruses: N75K (aspartic acid at position 75 of hemagglutinin protein is replaced by the lysine), N165K (aspartic acid is replaced by lysine at position 165) and S175P (serine replaced by proline at position 175) determined them into cluster B/Brisbane/60/2008-like viruses. And mutation that caused the L58P substitution (leucine replaced by proline at position 58) grouped all sequenced influenza viruses type B, isolated in Ukraine into single cluster, which included most of the selected isolates in this season (Fig.3.). Also Ukrainian isolate B/Odessa/3886/2010 was selected in London as the reference strain (Fig. 3.) among the viruses cultivated L58P substitution.

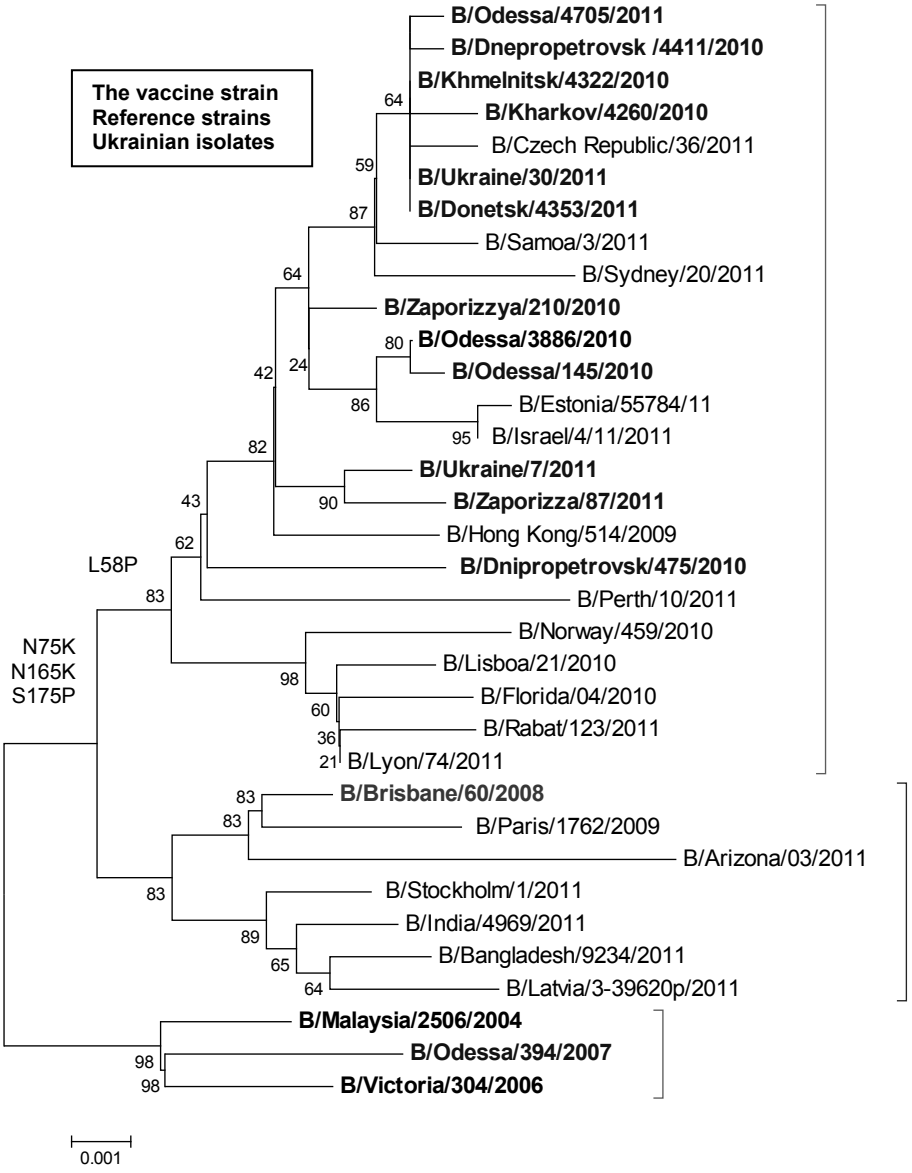


**Fig. 3. Phylogenetic tree of hemagglutinin(HA) genes of influenza viruses type B B/Victoria/2/87 line constructed by NJ method with Kimura 2-parameter model (Bootstrap values are indicated)**

**Phylogenetic comparison of hemagglutinin (HA) genesofinfluenza A (H3N2) viruses**

We have selected only two isolates belonged to A(H3N2) subtype:A/Khmelnitsky/1340/2010 and A/Ukraine/175/2011, isolated in our laboratory during the 2010-2011epidemic season. They amounted to 1% among the total number of influenza viruses isolated in Ukraine. This subtype of influenza A viruses was isolated in USA, Canada, Europe, Africa, Hong Kong and Argentina.

Phylogenetic analysis showed that virus isolated in Khmelnytsky has located near A/Brisbane/10/2007 and A/Wisconsin/67/2005 viruses – earlier representatives of this genetic branch. This mean that isolate was evolutionarily "old", and has preserved genetic similarity to viruses 2005 – 2007 years (Fig.4.). As for isolate A/Ukraine/175/2011, it located within A/Victoria/208/2009 cluster, which included the majority of influenza A(H3N2) viruses from this epidemic season. Isolate A/Ukraine/175/2011showed the greatest genetic similarity to viruses isolated in Brazil, Moldova, Russia, etc., which were joined into subclaster A/Pert/10/2010-like viruses (Fig. 4.).

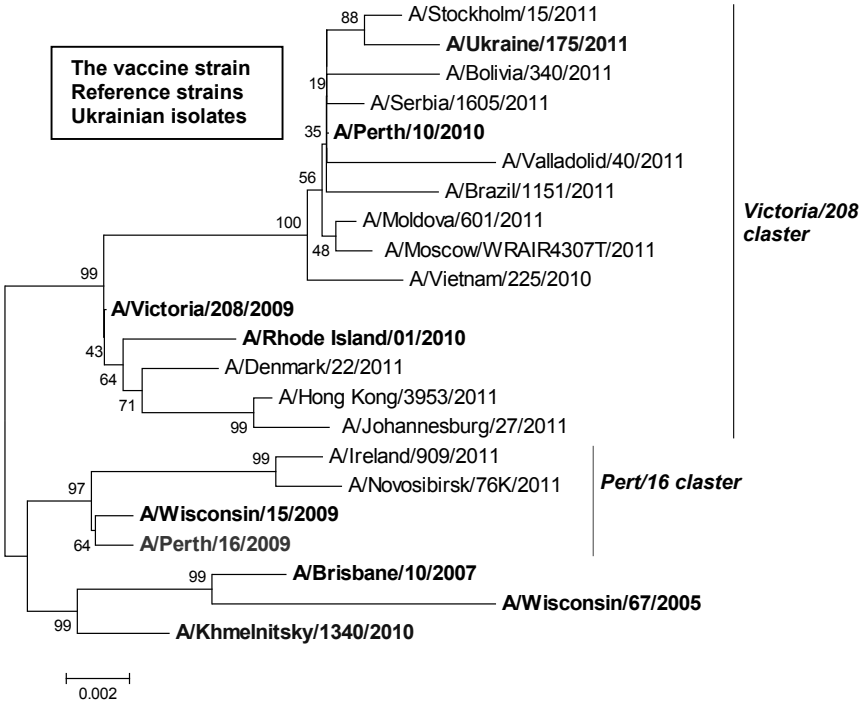


**Fig. 4. Phylogenetic tree of hemagglutinin(HA) gene influenza A(H3N2)viruses constructed by NJ method with Kimura 2-parameter model (Bootstrap values are indicated)**

Influenza A(H3N2)viruses, isolated during the 2010-2011 epidemic season in the world belonged to both Victoria and Pert clusters due to their genetic differences in the hemagglutinin. MutationsR261Q, E50K, P162S, N133D, R142G A212T and V213A were observed within A/Perth/16/2009 cluster, that differ viruses from A/Brisbane/10/2007. In cluster A/Victoria/208/2009 were observed next substitution in the hemagglutinin gene: N145S, V223I, N312S, D53N, Y94H, I230V, E280A and S312N (Fig.4.).

Hemagglutination inhibition assay showed that all Ukrainian isolates reacted in high enough titres with strainspecific serum to reference strains. It demonstrates their antigenic affinity to the vaccine strain A/Perth/16/2009.

**Conclusions.** The analysis of circulating influenza viruses in 2010-2011 epidemic season in Ukraine showed that epidemic was caused mainly by influenza viruses type B (up 55% of all selected viruses). Almost 45% of influenza isolates were similar to pandemic A(H1N1)sw strain. And only 2 isolates belonged to subtype A(H3N2).

Pandemic viruses A (H1N1)sw isolated in Ukraine were placed within two clusters, according their genetic structure. The amino acid substitutions N75K, N165K and S175P identified Ukrainian isolates type B as B/Brisbane/60/2008-like viruses. Phylogenetic analysis of influenza A(H3N2) viruses showed that isolate A/Khmelnitsky/1340/2010 located near A/Brisbane/10/2007 viruses – earlier representatives of this genetic branch. And virus A/Ukraine/175/2011 located within A/Victoria/208/2009 cluster, and showed the greatest genetic affinity to the A/Pert/10/2010-like viruses.All isolated in 2010-2011 influenza season viruses were antigenically related to vaccine strains.

1. http://platform.gisaid.org/ 2. Dan Graur and Wen-Hsiung Li. Fundamentals of Molecular Evolution: Second Edition. – Sinauer Associates. – 2000. 3. Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar SMEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. – 2011. 4. Saitou N. and Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees // Molecular Biology and Evolution. – 1987. – Vol.4. – P.406-425. 5. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences // Journal of Molecular Evolution. – 1980. – Vol.16. – P.111-120. 6. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap // Evolution. – 1985 Vol.39. – P.783-791. 7. Smith G., Vijaykrishna D., Bahl J., et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic // Nature. – 2009. – V. 459. – P.1122–1125. 8. Receptor specificity of subtype H1 influenza A viruses isolated from swine and humans in the United States / Chen LM, Rivailler P, Hossain J, Carney P. Virology. – 2011. – V. 412. – P.401-410. 9. http://ecdc.europa.eu/en/Pages/home.aspx

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**O. Mas, PhD student, O. Puhach, student, O. Andriychyk, PhD, V. Polischuk, Prof**

# FEATURES OF POLYPEPTIDE COMPOSITION OF PHAGES ISOLATED FROM ANTARCTIC SAMPLES OF SOIL AND MOSS ON DIFFERENT BACTERIA STRAINS – *XANTHOMONAS AXONOPODIS PV. BETICOLA 7325* AND *ERWINIA CAROTOVORA 216*

***Исследование морфологии и полипептидного состав фагов, выделенных из антарктических образцов почвы и мха на бактериях Xanthomonas axonopodis pv. beticola 7325 и Erwinia carotovora 216 IMV. Для исследуемых фагов, подобных морфологически, показана подобность и в полипептидном составе. Группа подобных фагов диференциировалась по составу минорных белков.***

***The morphology and polypeptide composition of phages isolated from Antarctic samples of soil and moss on bacteria X. axonopodis pv. beticola 7325 and E. carotovora 216 IMV were studied. For phages similar morphologically, similarities and polypeptide composition were shown. Group of similar phages differ in composition of minor proteins.***

**Introduction.**Even though bacteriophages play a major role in maintaining ecological balance of microbial communities, their potential influence on biogeographical patterns of bacterial communities remain greatly under-investigated. Features of their evolutionary relationships, adaptation to

bacteria hosts in extreme climatic conditions and functioning of such communities still require a detailed investigation [1].

One of the most important tasks is characterization of phages isolated directly from environment. Generalization of patterns will allow understanding the circulation of phages in nature, evolutionary changes in their populations within local regions.

However, Antarctic viral ecology is not sufficiently studied, there's virtually no dataon functioning of "phage-bacteria" system in ecological niches characterized with extreme conditions. Therefore, description of biodiversity phages and bacteria in Antarctic ecosystems and determining their ecological role is necessary [2].

The aim of this work was a comparative characterization of protein composition of phages which were isolated from Antarctic samples of soil and moss on bacterial strains *X. axonopodis pv. beticola* 7325 and *E. carotovora 216* IMV. An investigation of these phages characteristics is important to establish the range of phage propagation, to study evolutionary processes in phage populations and to analyze dependence of changes in the structure of phage reproduction on different hosts.

**Materials and methods**. Samples of soil and moss, which were selected during seasonal work on the Argentine Islands archipelago in locations Ukrainian Antarctic station "Akademik Vernadsky", were analyzed. Samples of soil and moss were selected from different areas of the islands. It were taken and kept in sterile plastic bags in the freezer. All further studies were conducted after delivery to Ukraine.

5 g of sterile material were taken and brought to 50 ml of 0.1 M Tris-HCl buffer. The filtrate was collected and bacteriophage particles were collected by ultracentrifugation at 100,000 x *g* for 2 h. The pellet was resuspended in 0.5 ml 0.1 M Tris-HCl buffer (pH 7.0).

Phages detected by direct inoculation. Titers were determined in plaque forming unit per ml (PFU/ml) by agar-layer technique by Gratia [3].

Preparations of concentrated phage were analyzed by TEM [4]. Electrophoretic characteristic of polypeptide composition of phages analyzed by Laemmlimethod using 14% resolving gel and 5% stacking gel, followed byCoomassie Brilliant Bluestaining [5]. Determination of molecular weight of proteins was performed using a set of marker proteins LMW (Fermentas): 116 kd – β-galactosidase from *E.coli*; 66,2 kd – Serum Albumin Bovine; 45 kd – Ovalbumin; 35 kd – Lactate dehydrogenase; 25 kd – REase Bsp98I from *E.coli*; 18,4 kd – β-Lactoglobulin from bovine milk, 14,4 kd – Lyzozyme from chicken egg white.

**Results and discussion.** Electron microscopy studies revealed that isolated phages fall into two morphological groups characterized with: i) isometric heads and long non-contractive tail (B1-morphotype, relates to the *Siphoviridae* family, isolated from moss and soil and accumulated on *X. axonopodis pv. beticola 7325;* isolates №3, 4, 8, 9); and ii) isometric heads and short non-contractive tail (C1-morphotype, relates to the *Podoviridae* family, order *Caudovirales,* accumulated on *E. carotovora 216*; isolate №11).

Polypeptide composition of phages is represented in Figure 1. Account of electrophoregram performed using the software "TotalLab TL120". The results of calculations are presented in Table 1.

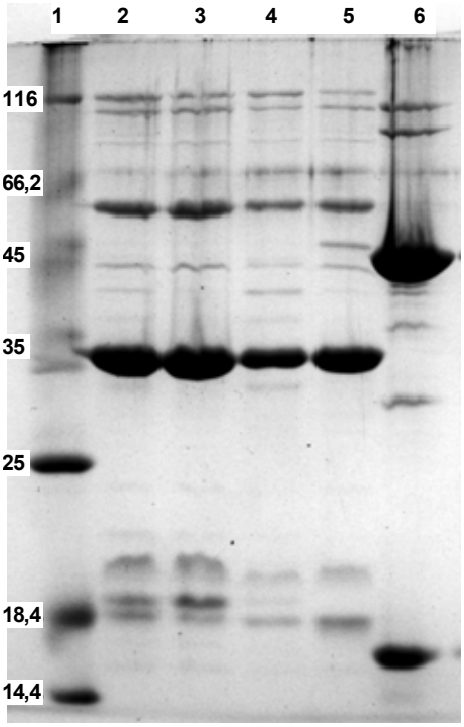


**Fig. 1. Electrophoregram of polypeptide separation of phages in the system Laemmli.**
Lanes: 1 – molecular weight marker LMW (Fermentas); 2, 3 – phages isolated from moss on sensitive bacterium *X. axonopodis pv. beticola 7325*, 4, 5 – phages isolated from soil on sensitive bacterium *X. axonopodis pv. beticola 7325*,
6 – phage isolated from soil on sensitive bacterium *E. carotovora 216*

***Table 1.*** **Molecular weight of phages polypeptides**

| | Molecular weight, kd | | | | | |
|---|---|---|---|---|---|---|
| Lane № / Band № | 1 | 2 | 3 | 4 | 5 | 6 |
| | Marker (kd) | Isolate 3 | Isolate 4 | Isolate 8 | Isolate 9 | Isolate 11 |
| 1 | 116 | 116,851 | 118,147 | 118,554 | 119,376 | 108,931 |
| 2 | 66,2 | 107,935 | 108,805 | 109,82 | 109,878 | 94,187 |
| 3 | 45 | 72,461 | 71,389 | 72,079 | 72,039 | 71,304 |
| 4 | 35 | 56,273 | 55,573 | 57,559 | 57,337 | 44,247 |
| 5 | 25 | 43,275 | 43,196 | 43,412 | 46,986 | 39,842 |
| 6 | 18,4 | 34,356 | 34,292 | 39,877 | 43,182 | 37,099 |
| 7 | 14,4 | 20,427 | 20,371 | 34,477 | 34,54 | 30,808 |
| 8 | | 19,139 | 19,081 | 32,128 | 20,208 | 16,873 |
| 9 | | 18,579 | 18,519 | 20,048 | 18,43 | |
| 10 | | | | 18,4 | | |

Research of protein composition of virions by electrophoresis system Laemmli allowed to set specific features among phages. Similarity of profiles identified polypeptides were characterized for all phages. Maybe, first passages on some bacteria-hosts influenced on phages. As seen in Fig. 1, phages formed two conventional groups according to the virions morphology and to the bacteria strains at which they were isolated (tracks 2, 3, 4, 5 – the first group; track 6 – the second group). Differences were observed mainly on the composition of minor polypeptides within the group of phages depending on thesource ofvirus isolation.Isolates №3 and №4 have a similar polypeptide composition by number of proteins and in molecular weight (may belong to the samebasicgroup) which differs from the polypeptide of isolates №8 and №9 thatwere isolatedfrom another source (moss).

A difference in composition of minorproteins within the group of phages suggests the impact of restriction – modification system or recombination processes in the host cells. In our opinion, the presence of minor differences in each studied phages may be result of mosaic theory of DNA phages evolution. Evolution of phages is associated with horizontal transfer of certain genetic modules [6]. Active phages, which take part in recombination exchanges, are source of genetic modules [7].

**Conclusions.**Phages, which were studied, have a high rate of similarity. Phages could get individual features by mutation or by exchanging DNA modules with other phages within their areal. In consideration of phages were isolated from nature, these results can reflect some evolutionary process in phage population.

1. Christian Winter, Markus M. Moeseneder, Gerhard J. Herndl, Markus G. Weinbauer. Relationship of Geographic Distance, Depth, Temperature, and Viruses with Prokaryotic Communities in the Eastern Tropical Atlantic Ocean // Microb. Ecol. – 2008. – V. 56.– p. 383–389. 2. David A. Pearce, William H. Wilson. Viruses in Antarctic ecosystems// Antarctic Science. – 2003. – № 15 (3). – P. 319–331. 3. Адамс М. Бактериофаги. – М.: Мир, 1961. – 527 с. 4. Миронов А.А., Комиссарчик Я.Ю., Миронов В. А. Методы электронной микроскопии в биологии и медицине: методологическое руководство. – СПб.: Наука, 1994. – 400 с. 5. Laemmli U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4 // Nature. – 1970. – V.227, №15. – p.608-685. 6. Hendrix R.W., Smith M.C.M. Burns. R.N., Ford M. E., Hatfull G.F. Evolutionary relationships among diverse bacteriophages and prophages: all the worlds a phage // Proc. Nat. Acad. Sci. USA – 1999 – V.96 – p. – 2192-2197. 7. Campbell A. Comparative molecular biology of lambdoid phages // Annu. Rev. Microbiol. – 1994 – V. 48 – p.193-222.

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**N. Matvienko, N. Kharkavliuk**

## FISH RNA VIRUSES

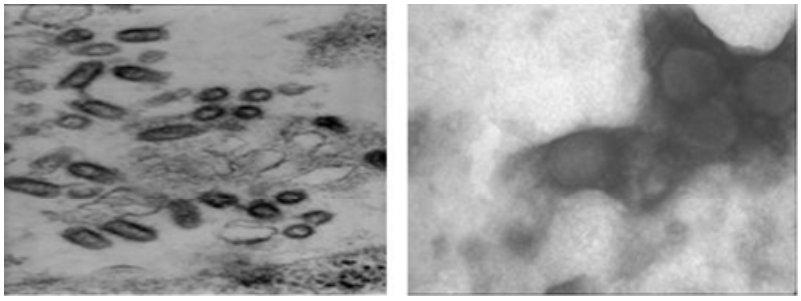
***Было проведено обследование рыб из разных ферм и водоемов Украины на наличие вирусной инфекции. Получены изоляты вирусов весенней виремии карпа (SVCV) и инфекционного панкреотического некроза (IPNV), а также изучены их биологическте свойства на культуре клеток. Для молекулярной диагностики SVCV, методом ПЦР, подобрана пара праймеров.***

***The fish from different farms and water bodies of Ukraine were observed for the presence of viral infection. Spring viraemia of carp virus (SVCV) and Infectious pancreatic necrosis* virus *(IPNV) were isolated and their biological properties were investigated. The pair of primers was designed for PCR identification of SVCV in fish clinical material.***

The main objectives of ichtyopatological research at this stage of development of Ukrainian aquaculture are studies of epizootic situation in fish farms in current environmental conditions, prevention of fish diseases and their early diagnostics based on modern techniques. Especially dangerous viral pathogens of fish are a group of RNA-containing viruses such as rhabdoviruses, which include the spring viraemia of carp virus (SVCV), virus haemorrhagic septicemia (VHS) virus and infectious hematopoietic necrosis of tissue (INN). It is important to mention also another representative of this group – aquabirnavirus, which causes salmon pancreas disease (IPNV) [1,2].

SVCV causes carp spring viraemia. The disease occurs as an epizootics type, is characterized by the development of septic process and ends with high fish mortality reaching 30-40% and sometimes 70% [3]. Reproduction of SVCV occurs in the endothelial cells of blood capillaries, hematopoietic tissue and nephrons. Infectious pancreatic necrosis virus was isolated from young rainbow trout (*Oncorhynchus mykiss*) held under intensive rearing condition. As his infection develops in trout, following pathological sings are recorded: disorders of motor coordination, skin darkening, significant pathological changes in the pancreas, liver, spleen and other parenchymal organs, and mortality of fish juveniles reaches 50-70%.

An optimal composition of the buffer was selected for preserving virus during its transporting to the laboratory. Electron microscopic investigations demonstrated that IPNV is an icosahedral-shaped virus appearing as a six-sided structure having 65-85 nm in diameter (*Fig.1*).



a b

**Fig.1. Transmission electron micrographs of the SVCV isolated from carp (*Cyprinus carpio*) (a) and IPNV isoladet from rainbow trout (*Oncorhynchus mykiss*) (b)**

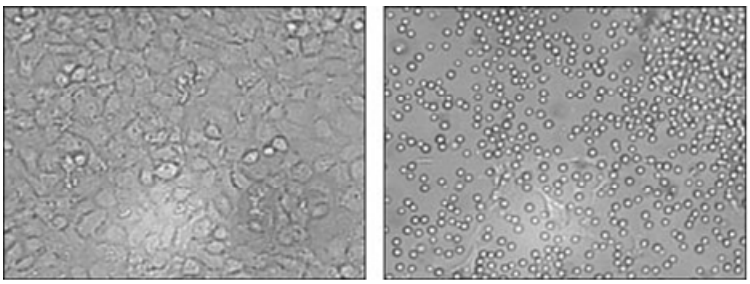
Virus identification was made using enzyme-linked immunosorbent assay (ELISA) on the TestLine systems and using PCR method (RT-PCR) [4].

**Material and methods.** The materials for virological studies were selected at fish farms and natural water bodies of different regions of Ukraine. Most viral isolates were isolated from carp fry andrainbow trout yearlings with signs of viral lesions. Isolation and identification of viruses were carried out with the aid of classical virology methods.

Isolates of SVCV and IPNV were identified and investigated. For PCR analysis, including RNA isolation, cDNA synthesis, amplification and detection, we used "Kit-A" and "Revertaza-L" (AmpliSensyTM, Russia)kits.The setting reaction was carried out according to manufacturer's protocols. Detection of results was performed by electrophoresis [5,6].

For the selection of the most efficient primers, we performed a comparative analysis of nucleotide sequences in the region, which encoded the RNA-dependent DNA polymerase and used the "Vector" software [7,8].

**Results and discussion.** Cultural properties of SVCV were investigated on cell lines EPC, BF-2 and FHM. A reproduction of the SVCV was accompanied by a distinct cytopatohenic action and led to the complete destruction of monolayer within 5-7 days, virus titers were thus $10^{6,4-7,5}$ $TCID_{50}$/0,1ml, optimum cultivation temperature is 19-22 °C (*Fig.2*).



a b

**Fig. 2.The cytopathology of investigated cell lines after infection by SVCV. Normal monolayer of EPC (a) and after infection with carp rhabdovirus (b)**

Studiers of IPNV cytopatohenic were performed on cell lines BF-2, FHM, RTG-2. The first morphological changes were observed within 24 h after inoculation. At the same time advancing complex degenerative processes that accompanied the advent of the cell vacuoles, granularity, cells retained elongated shape or rounded. Titer of virus was $10^{5.2-6.4}$ $TCID_{50}$/0,1ml (Fig3).

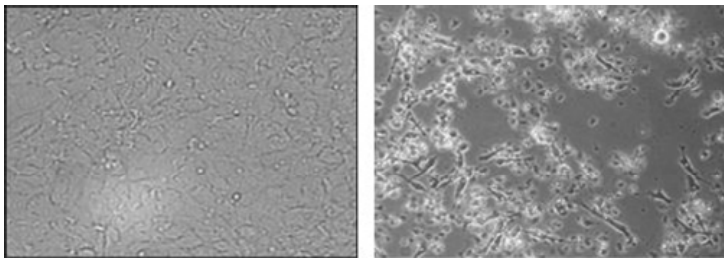


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**Fig.3. The cytopathology of investigated cell lines after infection by IPNV. Normal monolayer of FHM cells (a) and after infection with aquabirnavirus (b)**

When studying physical and chemical properties of SVCV, we found that the treatment with chloroform and warming up to 56 ºC results in completely loss of viral infectious activity. It was shown that the virus is resistant to pH ranging from 3.0 to 10.0.

Results of biochemical studies allow considering increase the formation of lipid peroxidation products in the body of a carp affected by SVCV as one of the links in the pathogenesis of the disease.

We matched pairs of primers that allow specific identification of SVCV in clinical material from fish. The comparative analysis of nucleotide sequences in the region encoding the RNA-dependent DNA-polymerase of birnaviruses. Primers for PCR synthesized from two conserved regions flanked by variable segment gene VP2 IPNV strain N1 (568-585 nd and 164-184 nd) through assumptions that our isolates belong to the European serotype.

Was investigated the biological characteristics of isolates of two major viruses of fish belonging to the group of RNA-containing viruses.

1. Lepa, Andrzej K. Siwicki, E. Terech-Majjewska Diagnostyka molekularna chorob wirusowych ryb hodowlanych. – Ochrona zdrowia ryb-aktualne problemy. Wyd. IRŁ Olsztyn, 2005- p/ 361-365. 2. Пичугина Т.Д, Завялова Е.А. Влияние вирусних инфекций на развитие аквакультуры в России // Ветеринарная медицина – №85-2005. – С.906-911. 3. Щелкунов И. С. Эпизоотическая ситуация по вирусным болезням рыб культивируемых рыб. Ветеринария №4, 2006. С 22-254. 4. Щелкунов, И.С., Щелкунова, Т.И., Купинская, О.А., Орешкова, С.Ф., Тикунова, Н.В., Ильичев, А.А. Гибридизационная тест-система для экспресс-диагностики весенней виремии карпа//Избранные труды ВНИИПРХ. – М., 2002. – Кн.1. – Т.2. – С.471-473. 5. Blake, S.L., W.B. Schill, P.E. McAllister, M.K. Lee, J.T. Singerand B.L. Nicholson. 1995. Detection and identification of aquatic birnaviruses by PCR assay. J. Clin. Microbiol. 33, 835-839. 6. Diagnostic Manual for Aguatic Animal Diseases 3 th Edition, 2000. 7. Suzuki, S., N. Hosono, R. Kusuda. Detection of aquati birnavirus gene from marine fish using a combination of reverse transcription- and nested PCR. J. Mar. Biotechnol. – 1997 – 5,205-209. 8. Joh, S.J., J.H. Kim, G.J. Heo. Genetic analysis of theVP2/NS junction region on segment A of marine Birnavirus isolated from cultured Flounder Paralichthys olivaceous. The J.Microbiol. – 2000. – 38, 44-47.

**Надійшла до редколегії 20.04.12**

**A. Mironenko, MD, A. Fesenko, PhD stud.**

## POLIOVIRUS INFECTION PROBLEMS IN UKRAINE

***Поскольку на сегодня прекращение циркуляции вирусов полиомиелита в мире остается актуальным вопросом, для контроля над состоянием защищенности населения Украины от полиовирусной инфекции, исследовано уровень антител в сыворотке крови здорового населения, а также проведен сбор данных по проведению вакцинопрофилактики у детей согласно с календарем прививок.***

***Since cessation of world's poliovirus circulation remain topical issue today, levels of antibodies in the blood serum of healthy population was investigated and was conducted data for vaccineprophylaxis in children. Those studies was developed for Ukrainian population security from poliovirus infection.***

Polio – an acute infectious disease characterized by general toxic symptoms and CNS injury by type of peripheral flaccid paresis and paralysis. Poliovirus, the causative agent of poliomyelitis, is a human ***enterovirus*** and member of the family of ***Picornaviridae***. There are three types of viruses: Type I (strain Brunhilde), type II (Lansing strain), type III (strain Leon). They are quite persistent in the environment.

Comprehensive study and scientific reasoning of the epidemiological surveillance system of poliomyelitis was the key to its eradication in most parts of the globe [1, 2].

But the problem of polio at present certainly not solved [3, 4]. This is evidenced by the epidemic, which in recent years there in the world and even in countries where for many years they were not registered [5, 6, 7].

For some infectious diseases, including polio, the main means of prevention is vaccination.

Maintaining the status of Ukraine as a territory free from polio depends primarily on the state of immunization of infection, as measured level of specific population immunity, and the percentage of people exposed to polio infection.

The **aim** of this work was to assess the levels of antibodies to the vaccine strains of polioviruses 1, 2, 3 types in human blood serum, as well as studies of the vaccineprophylaxis in Ukraine.

**Materials and methods**. Materials: serum of healthy people aged over 25 years living in different regions of Ukraine;data on vaccination in children from different regions of Ukraine. Methods: micromethod of neutralization reaction in cell culture Hep-2 [8].

***Results and discussion***. According to data obtained from regional SES Ukraine during 2009-2011., the analysis of the success of polio vaccination in children was conducted. Full vaccination complex involves the introduction of inactivated and oral polio vaccine to children less than one year, 18 months, revaccination at 6 and 14 years. Established in 2011, the percentage of children who received the vaccine was the lowest compared with 2009 and 2010. (Fig. 1.). It is shown that the level of polio vaccination of children is not satisfactory, because this figure should not be less than 95% for epidemiological welfare.

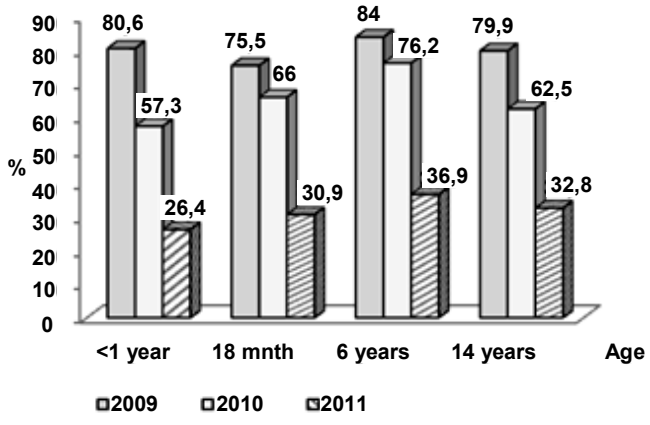


**Fig. 1. The status of vaccination in children in 2009-2011**

It was shown that index of antibodies to polioviruses 1, 2, and 3 types were at a sufficient level in children under 15 years. For example, 60.4% of children had sufficient antibody levels (1:16-1:64) for type 1 poliovirus (Fig. 2.). In type 2 polioviruses this figure was 61.4%, for type 3 polioviruses – 57.5%. However, high antibody titers (1:128-1:256 and above) to type 1 polioviruses was observed only in 29.9% of individuals, to type 2 polio virus – 28%, to 3 types – only in 14.5% of children.

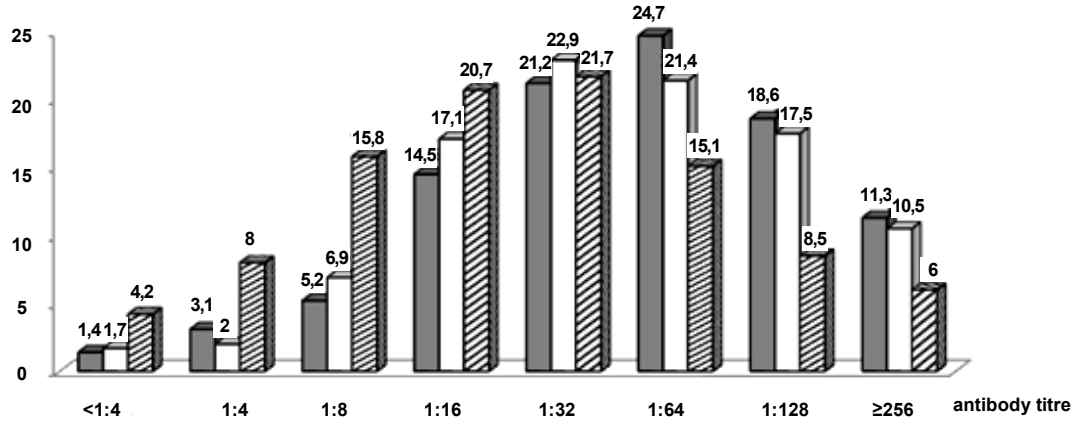


**Fig. 2.The antibody levels to three types of polioviruses in children in 2011**

For the study of immunity to polioviruses of all age population groups it was investigated serum of the adult population aged 25-65 years and older (Fig. 3.). Studies were conducted on cultured cells HEp-2 with vaccine strains of polio viruses 1, 2, 3 types and sera of subjects.

It is shown that the lowest levels of antibodies in the adult population were observed to poliovirus type 3. Thus, 67.2% of people were totally unprotected, 31.4% of subjects had low antibody titers (1:4-1:8), and only 1.4% – satisfactory titers. Among the samples of blood serum were not detected such, had high levels of antibody titers to type 3 poliovirus.

A somewhat different pattern was observed in the study of antibodies to poliovirus type 1. It is shown that 45.7% of the population were not protected (titer <1:4), were poorly protected 40% of those 34.4% had satisfactory antibody levels (1:16-1:64), the rest 2.8% – high levels of antibodies to polioviruses type 1.

For type 2 polioviruses 10% of the adult population of Ukraine appeared unprotected, the vast majority (47.2%) patients had low levels of antibodies to type 2 virus, the mean values of antibody titers were observed in 11.4% of people, and the smallest percentage (only 1.4 %) people had high levels of antibodies.

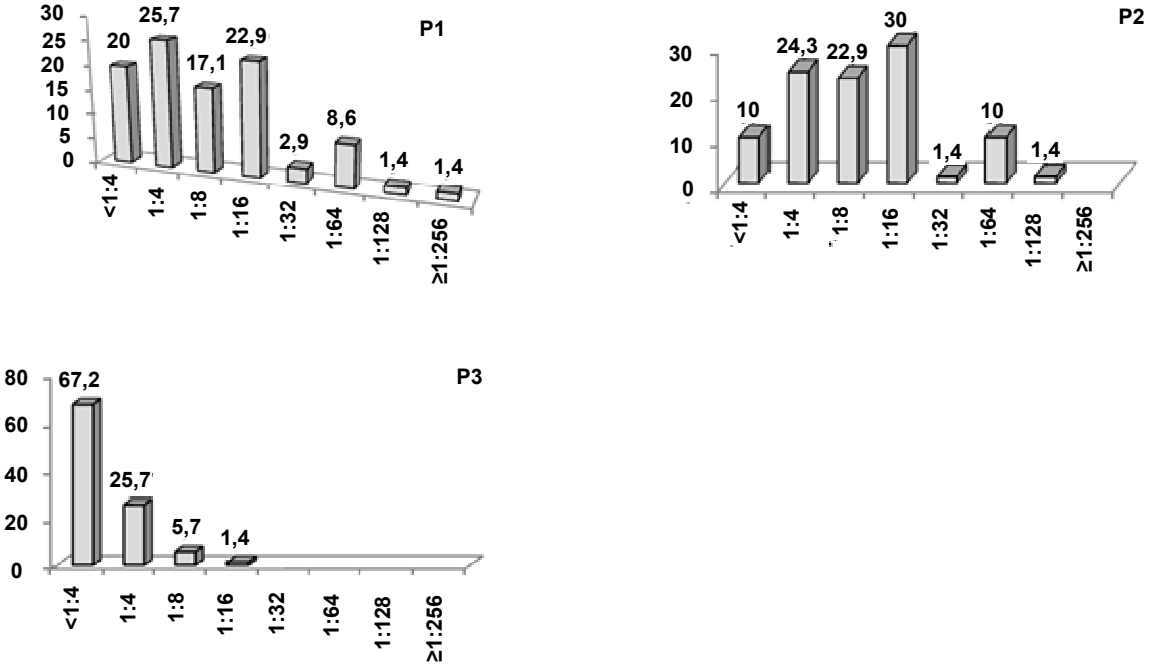


**Fig. 3. Status of immunity to polioviruses 1(P1), 2(P2), 3(P3) types in the adult population in 2011**

Thus, we can conclude unsatisfactory conduct vaccination among children and the general low level of antibodies against polio viruses of three types in the population of Ukraine. The first phenomenon can be explained by the failure parental cases of vaccination of their children, that had increasingly distributed nature among the population. Low levels of antibodies to polioviruses in the population of Ukraine, possibly due to the lack of circulation of wild strains of polio virus in the country. Subject to obtaining the full induced complex formed lasting immunity provided by memory cells. But if immune system does not meet with the agent for a long time, antibody levels accordingly reduced.

**Conclutions**. Thus, it was found that the level of vaccination to children under one year, 18 months., 6 years and 14 years, in accordance with the vaccination calendar in 2011 is much lower than in 2009-2010. However, levels of protective antibodies in children under 15 years in most cases had average levels (titre value within 1:16-1:64). Analysis of immunity to polio virus in the adult population in 2011. showed that persons aged 25-64 years and older

had low levels of antibodies to all three types of polioviruses, only a small percentage (1.4% -2.8%) of subjects had high titers of antibodies, antibodies to polio type were absolutely absent.

1.ЗадорожнаяВ.І., ДоанС.І., БондаренкоВ.І. Сучасна концепція епідеміологічного нагляду та профілактика ентеровірусних інфекцій. Матеріали доповідей науково-практичної конференції "Вчення Л.В. Громашевського у сучасних умовах боротьби з інфекційними хворобами". – Київ. – 2006. – С. 39-49. 2. D. Souza R., Kenett M. Australia declared polio free. // Commun. Dis. Intell. – 2002 – №26. – P.253-260. 3. Бондарев А.С. Можно ли считать полиомиелит ликвидированной инфекцией. Новости медицины. – 2010. – №330. – с. 18-62. 4. Малый В.П. Полиомиелит: современные проблемы. Клінічна імунологія. – 2010. – №4. – с. 57-62. 5. Эпидемиологическая справка ВОЗ №10-11. Завоз дикого полиовируса и ответные действия в Европейских регионах. http://www.euro.who.int/ru/what-we-do/healh-topics, 2011. 6. Poliomyelitis, Indonesia // Weekly epidemiology record – 2005. – V80. – № 23. – p. 201-202. 7. Polio this week. As of Wednesday 15 June 2011. Режим доступу http://www.polioeradication.org/Data 8.Руководство по лабораторным исследованиям полиомиелита. ВОЗЖенева, 1998. – 86 с.

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## DISTRIBUTION OF CACTUS VIRUS X IN SOME BOTANICAL GARDENS OF UKRAINE

***Проведено обследование растений семейства Cactaceae в коллекциях Никитского ботанического сада и ботанического сада Харьковского национального университета имени Н.В. Каразина. Описаны симптомы инфицированных растений. Свойства возбудителей исследовали с помощью растений-индикаторов, непрямого ИФА и трансмиссионной электронной микроскопии. Опираясь на серологические, биологические и морфологические характеристики изолированного вируса, мы можем предположить, что данный патоген является родственным X вирусу кактуса.***

***Screening of Cactaceae on virus diseases in the collections of Nikitsky Botanical Garden and Botanical garden of Karasin's Kharkiv National University has been conducted. Different symptoms were detected on virus-infected plants. To define properties of the indicated pathogen methods of host assay, indirect ELISA and transmission electron microscopy were employed. Basing on serological, biological and morphological properties, we suggest that isolated virus is related to Cactus virus X.***

**Introduction**.The culture of cactuses in modern floriculture occupies one of leading places. In accordance with literary data about 10 viruses are able to affectthe members of *Cactaceae* family: *Cactus virus X, Schlumbergera virus X, Opuntia virus X, Zygocactus montana virus X, Saguaro cactus virus, Sammons' Opuntia virus, Cactus virus 2 (CV2), Cactus mild mottle virus, Impatiens necrotic spot virus* and *Tomato spot wilt virus* [3,4]. Among them *Cactus virus X* is one of most dangerous. *Cactus virus X* is transmitted by mechanical inoculation, grafting and by contact between plants. [1,5,6]

**Materials and methods.** We have been collected plants with virus-like symptoms: *Mamillaria microhelia, Ausrocylindropuntia tunicata, Monvillea sp., Sulcorebutia sp., Bolivicereus samupatanus, Opuntia sp., Ferocactus sp., Thelocactus chrenbergii, Trichocereus bridgesii, Mamillaria magnimamma, Agave furcrea, Opuntia sp., Cereus sp., Astrophytum capricorne, Cereus sp., Astrophytum myriostigma, Astrophytum myriostigma v. nudum* from greenhouse collection of Nikitsky Botanical Garden and *Ritterocereus pruinosus* from the collection of Karazin' Botanic Garden of Kharkiv National University. Infectivity of disorders was confirmed proved using indicators plants typical for majority cactus viruses such as *Chenopodium murale, Datura stramonium, Gomphrena globosa, Nicotiana rustica*. Virus identification was carried out using indirect ELISA [2]. Same samples of plants of *Cactaceae* family were analyzed in electron microscopy at 30,000 magnification. [7]. Electrom microscopic examination we conducted with an Jem electron microscope. Crude sap preparations were negatively stained with 2% uranyl acetate.

**Results and discussion.** Cactus plants demonstrated different symptoms of virus infection. On some plants (*Mamillaria microhelia, Ferocactus sp., Thelocactus chrenbergii, Trichocereus bridgesii, Mamillaria magnimamma, Agave furcrea, Opuntia sp., Cereus sp., Astrophytum capricorne, Cereus sp., Astrophytum myriostigma* from greenhouse collection of Nikitsky Botanical Garden and *Ritterocereus pruinosus* from the collection of Karazin' Botanic Garden of Kharkiv National University) we observed mosaic symptoms, on other plants (*Ausrocylindropuntia tunicata, Monvillea sp., Sulcorebutia sp., Bolivicereus samupatanus, Opuntia sp., Astrophytum myriostigma v. nudum* from greenhouse collection of Nikitsky Botanical Garden) – symptoms of necrosis (Fig.1,2).

**Fig.1. Necrosis on the stem *Astrophytum myriostigma v. nudum***

**Fig.2. Mosaic on the stem *Ferocactus sp.***

To define biological properties of the pathogens, we conducted a host assay using indicator plants. Indicator plants were inoculated with sap obtained from cactus plants demonstrating virus-like symptoms. Necrotic local lesions observed on *Chenopodium murale* and *Gomphrena globosa* were typical for Cactus virus X (CVX) [5,6]. In indirect ELISA we observed positive reactions with sap *Mamillaria microhelia, Sulcorebutia sp., Bolivicereus samupatanus, Opuntia sp., Ferocactus sp., Mamillaria magnimamma, Opuntia sp., Cereus sp., Astrophytum capricorne, Astrophytum myriostigma* and *Ritterocereus pruinosus* with antiserum to Potato virus X (PVX, which is serologically related to Cactus virus X).

Comparing the results of bioassay and ELISA tests we further focused on samples of *Mamillaria microhelia, Sulcorebutia sp., Bolivicereus samupatanus, Opuntia sp., Ferocactus sp., Mamillaria magnimamma, Opuntia sp., Cereus sp., Astrophytum capricorne, Astrophytum myriostigma* from collection of Nikita Botanical Gardenand *Ritterocereus pruinosus* from the collection of Karazin' Botanic Garden of Kharkiv National University. These plants were probably infected with CVX. To confirm our assumption about CVX infection and to study the morphology of the pathogen we carried out transmission electron microscopy. In sap of all plants we registered filamentous virions with size 580×13 ± 2 nm (Fig.3), which is typical for *Cactus virus X.*

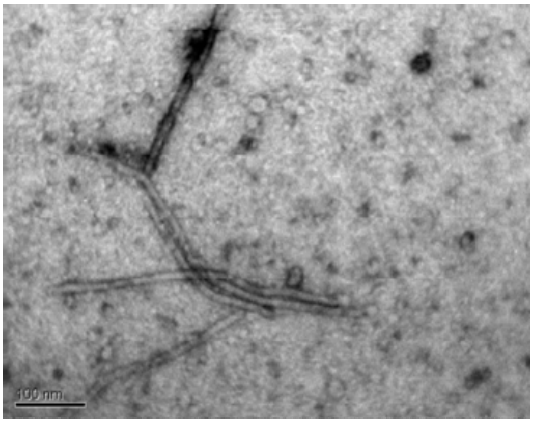


**Fig.3. Electron micrograph of flexible particles from plant material from *Ferocactus* sp.**

Summarizing the obtained results it is possible to assert that collectionsof Nikitsky Botanical Garden and Botanical garden of Karasin's Kharkiv National University were contaminated by Cactus virus X. Exchange collection material without testing on virusinfection, can increases chances of uncontrolled distributionof viral infections. Some infections are symptomless. In this case the asymptomatic virus infectionin cactus makes a danger because these plants can become thesource of distribution pathogens in collections. In addition,cactuses could support reproduction of viruses other typesof plants and, thus, be the reservoirs of plant virus infections.A timely exposure and permanent control of the stateof population of these cultural plants is the obligatory link ofthe system of their protecting from this group of pathogens.

1. M. Chessin, D. Lesemann. Distribution of Cactus Viruses in Wild Plants// Phytopatology. – 1972. – Vol.62. – P.97-99. 2. L.M.L. Duarte, M.A.V. Alexandre, E.B. Rivas, R. Harakava, S.R. Galleti and M.M. Barradas POTEXVIRUS DIVERSITY IN CACTACEAE FROM SÃO PAULO STATE IN BRAZIL Journal of Plant Pathology (2008), 90 (3), 545-551. 3. Virus Taxonomy Classification and Nomenclature of Viruses Ninth Report of the International Committee on Taxonomy of Viruses Editors Andrew M.Q. King, Michael J. Adams, Eric B. Carstens, and Elliot J. Lefkowitz International Union of Microbiological Societies Virology Division Elsevier academic press. 4. R. Koenig, C.W.A. Pleij, S. Loss, W. Burgermeister, H. Aust, J. Schiemann. Molecular characterization of potexviruses isolated from three different genera in the family Cactceae // Arch. Virol. – 2004. – №149. – P.903-914. 5. J.R. Lastra, Daisy Gaskin, y R.C. de Uzkątegui. Virus x del cactus en Venezuela//Agronomia Tropical.1976. – №26. – P.303-310. 6. Liou MR, Hung CL, Liou RF (2001) First report of Cactus virus X on Hylocereus undatus(Cactaceae) in Taiwan. Plant Dis 85: 229. 7. G.M. Milbrath, M.R. Nelson and R.E. Wheeler. The Distribution and Electron Microscopy of Viruses of Cacti in Southern Arizona// Phytopatology. – 1973. – №63. – P.1133-1139.

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## ELECTRON MICROSCOPY AND BIOLOGICAL PROPERTIES OF THE PATHOGEN AFFECTING *PLATANTHERA BIFOLIA*

***Проведено обследование больных растений Platanthera bifolia, отобранных в Канивском природном заповеднике. Была доказана инфекционность сока, а также возможность механической передачи вируса. Методом трансмиссионной электронной микроскопии в образцах пораженных растений были выявлены сферические вирусоподобные частицы, диаметром около 50 нм. Согласно морфологическим особенностям, вирус, выделенный из растений Platanthera bifolia, может принадлежать к семейству Caulimoviridae.***

***The plants of Platanthera bifolia collected in Kaniv National Reserve were observed. The infectivity of plant sap and viral capability to transmit in mechanical manner were confirmed. The spherical particles with diameter about 50 nm were observed in preparations from symptomatic leaf tissue in transmission electron microscope. According to viral morphology the pathogen of Platanthera bifolia may be a member of Caulimoviridae.***

**Introduction.***Orchidaceae* Juss. is one of the biggest families of flowering plants which includes about 35 000 species. They can be found all over the world excluding deserts and Polar Region [2]. Viral diseases of orchids are known from the middle of XX century [4]. Until now more over 30 viruses of orchids have been described [8, 10]. The majority of these viruses were detected as pathogens of tropical orchids which are cultivated *in situ*. On the other hand the viruses of terrestrial orchids of temperate zone are studied not enough. The infection of *Cypripedium* sp., *Orchis sp.*, *Ophrys* sp. by T*obacco rattle* (TRV) and T*urnip mosaic virus* (TuMV) were described [5]. Also the antigens

to A*rabis mosaic virus* (ArMV)*, Bean yellow mosaic virus* (BYMV)*,Tobacco mosaic virus* (TMV),*Tomato aspermy virus* (TAV), TRV and TuMV were detected in orchids of natural Ukrainian flora [1].

**Materials and methods.** Plant material was collected in Kaniv Natural Reserve (Snakes' Islands). For further investigation we used plants of *Platanthera bifolia* with typical viral symptoms (fig. 1).

**Figure 1. Necrotic lesions on the leaves of *Platanthera bifolia***

Virus identification was carried out using standard indirect ELISA and DAS-ELISA with polyclonal antiserums to *Cucumber mosaic virus* (CMV), *Tomato spotted wilt virus* (TSWV), *Potato virus Y* (PVY), BYMV, TAV, TMV, TRV, TuMV and *Cauliflower mosaic virus* (CaMV).

Biological properties of viruses were studied using following test-plants: *Chenopodium murale, Gomphrena globosa, Lycopersicon esculentum, Nicotiana alata, N. benthamiana, N. rustica, N. tabacum, Petunia hybrida* and *Zinnia elegans.* The test-plants were inoculated in early stages of growth by mechanical sap transmission, applying carborundum as an abrasive. The inoculum was prepared by homogenizing plant tissue with 0.1 M phosphate buffer (pH 7.4) and followed by centrifugation (5000 rpm for 20 min).

The morphology of virions were studied in leaf dip preparations negatively stained with 2% uranyl acetate EM using a JEM-1400 electron microscope, magnification 60 000.

**Results and discussion.** The ELISA testing revealed no antigens of BYMV, CMV, TAV, TMV, TRV, TSWV, TuMV and PVY in samples of infected *P. bifolia.*

The sap of necrotic leaves from *P. bifolia* were experimentally inoculated to the range of test-plants, common for many polyhostal viruses [3, 7]. All inoculated plants show no local reaction but *L. esculentum, N. benthamiana, N. rustica* and *N. tabacum* reacted systemically. The systemic symptoms of viral infection appeared two months after inoculation. No visual symptoms were observed on *C. murale, G. globosa,P. hybrida* and *Z. elegans* (table 1).

***Table* 1. Reaction of-test plants inoculated with sap from *Platanthera bifolia***

| Test-plant | Symptoms |
|---|---|
| *Chenopodium murale* | -/-* |
| *Gomphrena globosa* | -/- |
| *Lycopersicon esculentum* | Chlorotic lesions, leaf deformation |
| *Nicotiana alata* | -/- |
| *Nicotiana benthamiana* | Mosaic |
| *Nicotiana rustica* | Mosaic |
| *Nicotiana tabacum* | Chlorosis |
| *Petunia hybrida* | -/- |
| *Zinnia elegans* | -/- |

*"-/-" – no visual symptoms

EM examination of infected plants tissue revealed the presence of spherical virus-like particles about 50 nm in diameter (fig. 2). Such morphology are typical for representatives of *Caulimoviridae*: *Caulimovirus, Cavemovirus, Petuvirus, Soymovirus* [9].

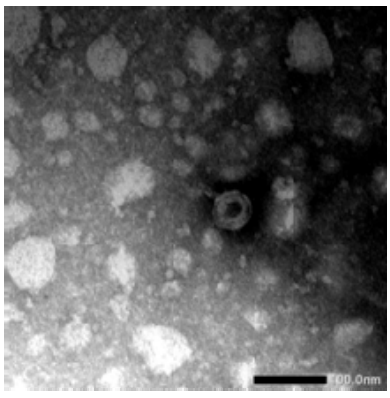

**Figure 2. Electron micrograph of virus-like particles in the sap of infected plants**

The majority of viruses belonging to the listed genera can be transmitted by mechanical inoculation. The reaction of test-plants eliminates such viruses as *Blueberry red ringspot virus*, *Cestrum leaf curling virus*, *Petunia vein clearing virus* and *Tobacco vein clearing virus* which are not transmitted in mechanical manner [3, 6].

For further identification of virus DAS-ELISA test were used. No positive reaction with antiserum to CaMV was obtained. According to results of DAS-ELISA we can exclude infection of *P. bifolia* by CaMV and serologically related viruses: *Carnation etched ring virus*, *Dahlia mosaic virus*, *Horseradish latent virus* and *Strawberry vein banding virus* [3].

In conclusion, host assay of sap samples extracted from infected plants of *P. bifolia* approved its infectivity. EM studies confirmed the presence of virus-like particles in infected plant tissues. The isometric morphology and size range allows to suggest that the viral pathogen of *P. bifolia* can be a representative of family *Caulimoviridae*. The cases of similar virus infection of orchids are not described in literature data. However, there are not much investigation about spreading viruses in natural ecosystems. Further work will be focused on identification of the virus infecting *P. bifolia*.

1. Коротєєва Г. В.,Поліщук В. П. ВірусизозулинцевихприродноїфлориУкраїни // Мікробіол. Журн. – 2004. – Т. 66 – № 2. – С. 74-80. 2. Черевченко Т.М. Тропические и субтропические орхидеи/ К.: Наукова думка. – 1993. – 253 с. 3. Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L. and Zurcher, E.J. (eds.) (1996 onwards). 'Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 20th August 1996. 4. Jensen D.D. Mosaic of Cymbidium orchid // Phytopathology. – 1950. – Vol. 40. – P. 966-967. 5. Lesemann, D.E., Vetten, H.J. The occurrence of tobacco rattle and turnip mosaic virus in *Orchis ssp*., and of an unidentified potyvirus of *Cypripedium calceolus* // Acta Hortic. – 1985. – Vol.64. – P.45-54. 6. Lockhart B. E., Menke J., Dahal G., Olszewski N. E. Characterization and genomic analysis of tobacco vein-clearing virus, a plant pararetrovirus that is transmitted vertically and related to sequences integrated in the host genome. // Journal of General Virology – 2000. – Vol. 81. – P.: 1579–1585. 7. Navalinskienė M., Samuitienė M. Dekoratyvinių augalų virusinės ligos ir jų sukėlėjai Lietuvoje. Monografia. – Kaunas:Lutute, 2006, – 256 p. 8. Orchid biotechnology II // eds. Chen H.-W. and Chen H.-H. – World Scientific Publishing Co Pte Ltd, Singapore. – 2011. – 380 p. 9. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses / King A. M. Q., Adams M. J., Carstens E.B., Lefkowitzl E.J. – NewYork: Elselver Academic Press., 2012. 10. Zettler F.W., Wistler G.C., Elliot M.S., Ko N.-J. Viruses of orchids and their control // Plant. Disease. – 1990. – Vol. 74. – P. 621-626.

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**J. Polák**

# BIOTECHNOLOGICAL (GM) CROPS IN THE WORLD AND EUROPE, PLUM HONEYSWEET RESISTANT TO PPV, RESEARCH IN THE CR: THE PAST, THE PRESENT AND THE FUTURE

***Быстрое увеличение площадей посевов ГМ-культур, делает их наиболее высокоадаптивными посевными культурами в истории сельского хозяйства. Здесь представлены результаты девяти лет тестирования слив клона С5 (трансформирован капсидным белком вируса шарки сливы) при высокой и постоянной инфекционной нагрузке PPV-Rec, как самостоятельного агента, так и в сочетании с вирусами карликовости чернослива и кольцевой хлоротичности яблони.***

***Fast increasing hectarage of GM crops makes them the highly adopted crop technology in the history of agriculture. Results of nine years testing of plum clone C5 (transformed with the Plum pox virus coat protein) under the high and permanent infection pressure of PPV-Rec alone and in combinations with Prune dwarf virus, and Apple chlorotic leafspot virus both from graft inoculation and natural aphid vectors are presented.***

**Introduction.** Research in genetic engineering started in late seventies (Colwell et al., 1985). The first genetically modified (GM), syn. biotechnological (Biotech) crops were commercialized in 1995 (cotton, company Monsanto; potato, company Syngenta). 1.7 million hectares of Biotech crops (cotton, corn, potato) were planted in 1996 already (USDA-APHIS Biotechnology Permits Database, available at htpp://www.nbiap.vt.edu/ (March 2001). Increase in hectarage to 160 million hectares in 2011 (Fig. 1) makes Biotech crops the fastest adopted crop technology in the history of agriculture. The International Service for the Acquisition of Agri-biotech Applications (ISAAA) is every year publishing "Global status of commerialized Biotech/GM crops". The last one was published as ISAAA Brief No. 43 in 2011 (James C., 2011). The objective of this Brief is to provide information and knowledge to the scientific community and society on Biotech/GM crops and its contribution to global food, feet, fiber and fuel security, to sustainable agriculture.

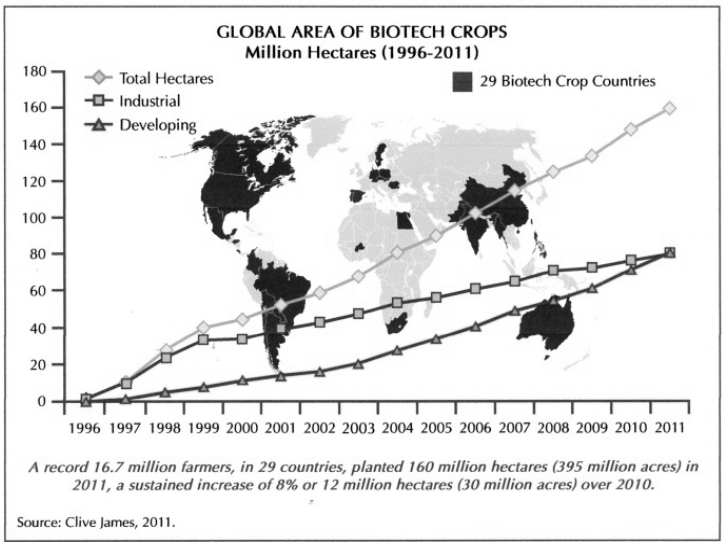


**Fig. 1. Global area of biotech crops**

Of the 29 countries planting Biotech crops in 2011 (Fig. 2), 19 were developing countries and 10 were industrial countries. 60% of the world's population live in countries planting Biotech crops. The US is the lead producer of Biotech crops with 69 million hectares. US is followed by Brazil with 30.3 mil. ha, Argentina (23.7 mil. ha), India (10.6 mil. ha), and Canada (10.4 mil. ha). Developing countries grew close to 50% of global Biotech crops. Another five countries, China, Paraguay, Pakistan, South Africa, and Uruguay each grew more than 1 million hectares. Biotech soybean remains with 75.4 million hectares (47% of global Biotech area) the dominant crop, followed by Biotech maize with 51 million hectares, Biotech cotton 24.7 mil. ha (Fig. 3), and Biotech canola 8.2 mil. ha.

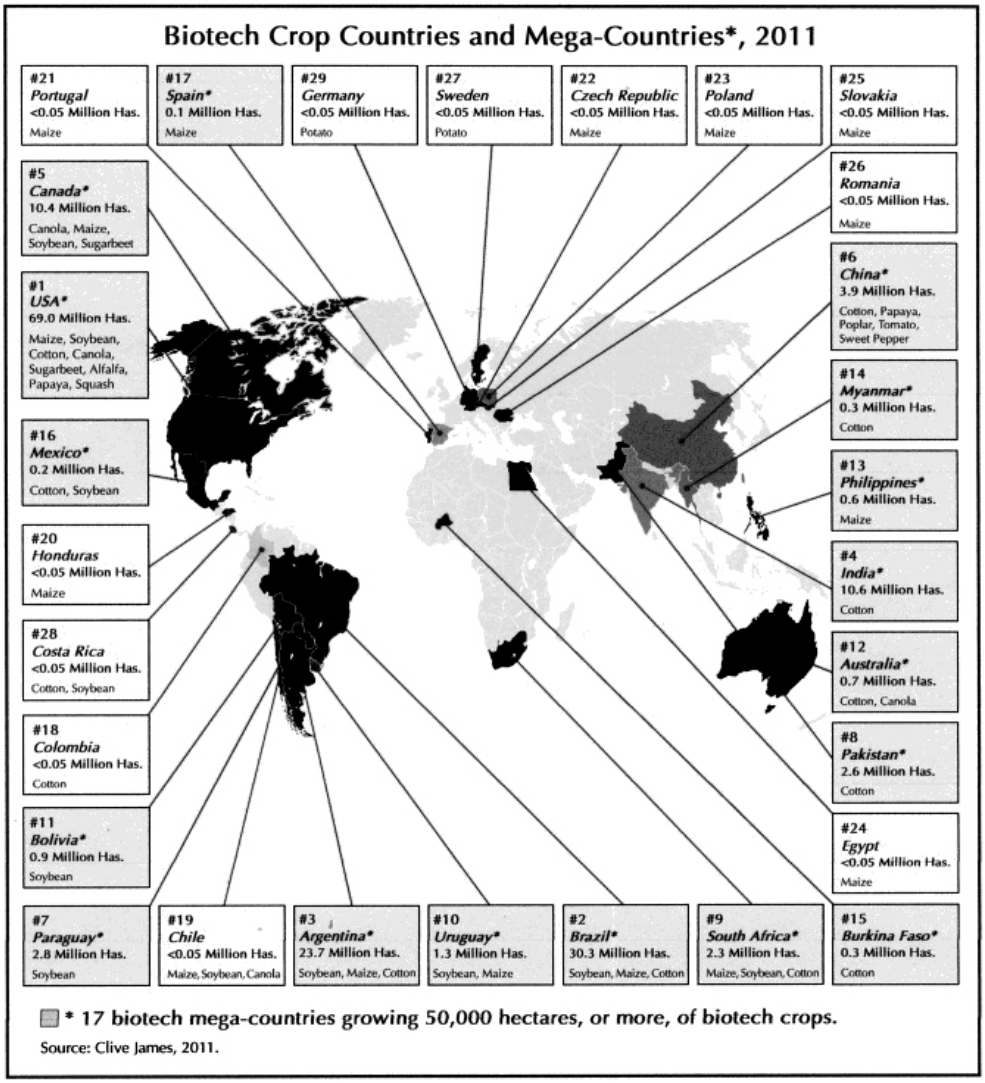


**Fig. 2. Global map of biotech crop countries and mega-countries in 2011**

**Fig. 3. Biotech cotton in grown in Africa**

The only 0.114507 million hectares of Biotech crops were planted in Europe. Six EU countries, Spain, Portugal, Czechia, Poland, Slovakia, and Romania planted 114490 ha of Bt maize. Sweden (15) and Germany (2) planted 17 ha of Biotech starch potato cv. 'Amflora'. Spain as the first European country is with 97326 ha of Bt maize on the 17. place. In the Czech Republic the Bt maize was grown on 5091 ha in 2011. European policy in relation to GM crops is scandal. In October 2011, forty one leading Swedish biological scientists in a strongly-worded open letter to politicians and environmentalists asked to revise European legislation to allow society to benefit from Biotech crops. A contingent of scientists from the United Kingdom endorsed the Swedish petition.

Biotech crops are strongly contributing to food security. This was achieved from 1996 to 2010 (in five years) by: increasing crop production and value by US 78 billion dollars, providing a better environment, by saving 443 million kg a.i. of pesticides; in 2010 alone reducing $CO_2$ emissions by 19 billion kg, equivalent to taking 9 million cars off the road, saving 91 million hectares of land, and helping 15 million farmers, the poorest people in the world. From the point of food security European policy is going against humanity in the world.

Plum (*Prunus domestica* L), clone C5 transformed with the *Plum pox virus* (PPV) coat protein (CP) was obtained by Scorza et al. (1994). Clone C5 (cv. 'HoneySweet' at present) was proved to be highly resistant to PPV under glasshouse conditions (Ravelonandro et al., 1997). Field tests were conducted in Poland (Malinowski et al., 1998), Romania (Ravelonandro et al., 2002), Czech Republic (Polák et al., 2005), and in Spain (Malinowski et al., 2006). The all experiments confirmed the resistance of clone C5 to PPV infection. A trial of high and permanent infection pressure of PPV-Rec alone and in combinations with *Prune dwarf virus* (PDV), and *Apple chlorotic leafspot virus* (ACLSV) was initiated in the Czech Republic and published (Polák et al., 2008a; 2008b). Summarized results of nine years testing of plum clone C5 under the high and permanent infection pressure both from graft inoculation and natural aphid vectors are presented. The transgenic plum trees were evaluated during the years 2003-2011.

**Materials and Methods.**

**Field trial, transgenic plum trees, inoculation of viruses.**

Plum clone C5 buds were grafted onto virus-free rootstocks of St. Julien in 2002, and 55 trees of P. domestica clone C5/St. Julien were obtained. Eleven trees inoculated with PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV, control non-inoculated trees were obtained and a plantation established (Fig. 4). PPV-Rec, ACLSV, and PDV infected buds were allowed to grow throughout the period of evaluation. The transgenic clone C5 part of each tree was under a very strong inoculum pressure.

**Fig. 4. Plantation of HoneySweet trees in CR in 2011 (Orig. J. Polák)**

**Evaluation of leaf and fruit symptoms, quality of fruits.**

The all trees were evaluated every year from May to September for the presence of viral symptoms in leaves (2003-2011). Fruit symptoms were evaluated in July and August 2010 and 2011 before and at ripening. In 2010 and 2011 overall fruit uniformity, attractiveness, weight, length, width and fruit thickness, flesh thickness, fruit shape, skin colour, flesh colour, flesh firmness, flavour, flesh freeness, total soluble solids, total titratable acidity, stone size, weight and stone/flesh ratio, and dry weight of fruits harvested from trees of clone C5 inoculated with PPV-Rec, and unicoculated control trees of clone C5, and 'Stanley' were recorded.

**Serological detection of viruses.**

ELISA testing of leaves was performed every year in June. Fruits were evaluated in August 2010. Polyclonal antibodies raised against PPV, ACLSV, and PDV (Bioreba, Switzerland) were used in DAS-ELISA (Clark and Adams, 1977). Leaf samples were extracted in phosphate-buffered saline. The relative concentration of PPV-Rec was determined by semiquantitative DAS-ELISA in samples prepared from symptomatic leaves in June 2005 and 2007, and in samples prepared from fruits in August 2010. The relative concentration of PPV protein was established by determining the lowest dilution of leaf or fruit sample with positive reaction in semiquantitative DAS-ELISA (Albrechtová et al., 1986).

**Detection of viruses by Reverse Transcription-Polymerase chain reaction (RT-PCR).**

100 mg of ground leaf or fruit tissues were used for total RNA extraction by using RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the procedure recommended by the manufacturer. PPV-Rec was detected by RT-PCR using primer pair mD5/mM3 as described by Šubr et al. (2004). For PDV and ACLSV the primers as described by Jarošová and Kundu (2010) were used.

**RESULTS AND DISCUSSION.** No PPV symptoms appeared in the leaves of transgenic plum clone C5-cv. 'HoneySweet' trees in the first year after inoculation with PPV-Rec by graft inoculation. PPV symptoms appeared only in the leaves that emerged from infected buds (IB). Mild diffuse spots and rings appeared two years after inoculation in some basal leaves of cv. 'HoneySweet' trees inoculated with PPV-Rec, and in those inoculated with virus

combinations PPV-Rec + ACLSV, PPV-Rec + PDV, and PPV-Rec + ACLSV + PDV (Polák et al., 2005). PPV presence was confirmed by ELISA and RT-PCR. A reduction of symptoms was observed beginning in the third year after virus inoculation. PPV symptoms were observed only in several basal leaves and symptoms were milder in each year (Polák et al., 2008a). Further reduction of PPV symptoms was observed during the vegetation period from June to September. No differences in the intensity of PPV leaf symptoms among different virus combinations were observed in the years 2003-2011. No symptoms of PDV and ACLSV appeared during the vegetative periods of 2003-2011. PDV was not detected by ELISA in transgenic parts of trees inoculated with PPV-Rec + PDV and PPV-Rec + PDV + ACLSV. The presence of PDV was inconclusive by RT-PCR. PDV was detected by ELISA and RT-PCR only in leaves growing from the IB. ACLSV was detected by ELISA and RT-PCR in leaves of transgenic parts of trees inoculated with PPV-Rec + ACLSV and PPV-Rec + PDV + ACLSV. No symptoms of PPV, PDV, and ACLSV appeared in the leaves of non-inoculated control trees of cv. 'HoneySweet' throughout the experiment. PPV, PDV, and ACLSV were not detected in control trees by DAS-ELISA and RT-PCR. The growth of control trees was more vigorous in comparison with trees inoculated with PPV, and combinations with PDV and ACLSV. The severe PPV symptoms which appeared first in 2003 in IB leaves growing from buds infected with PPV-Rec appeared again every year (2004-2011) with the same intensity.

The relative concentration of PPV-Rec in symptomatic leaves of cv. 'HoneySweet' determined by semiquantitative DAS-ELISA fluctuated from $1.56x10^{-2}$ to $9.76x10^{-4}$ in 2005, and from $5.0x10^{-1}$ to $7.81x10^{-3}$ in 2007. There were no significant differences in relative concentration of PPV among combinations of inoculated viruses.

Pomological evaluation of external and internal characteristics of fruits harvested from non-graft-inoculated cv. 'HoneySweet' trees, cvs. 'Stanley', and 'Domácí švestka' trees, and from cv. 'HoneySweet' trees growing nine years under the high and permanent infection pressure of PPV-Rec, PPV-Rec + PDV, PPV-Rec + ACLSV, PPV-Rec + ACLSV + PDV demonstrated the high quality of 'HoneySweet' fruits.

Two-year results indicate that the characteristics of 'HoneySweet' fruits harvested from control virus non-inoculated trees are well within the range of the characteristics of control cultivars 'Stanley' and 'Domácí švestka' and are of higher quality in some characteristics. Fruit harvested from 'HoneySweet' trees inoculated with PPV-Rec + ACLSV + PDV, PPV-Rec + PDV, PPV-Rec + ACLSV, and PPV-Rec were comparable with fruits from control healthy 'm 'HoneySweet' trees indicating that there was little, if any, effect of the virus inoculations on fruit quality of 'HoneySweet'.

'HoneySweet' plum trees resistant to PPV remained virus-free under natural aphid-vectored infection pressure throughout this nine-year study. Graft inoculated trees were exposed to a very high infection pressure with IB being allowed to reach the size of 20-30% of the supporting 'HoneySweet' tree (Fig. 5a, b). Under this high and permanent virus pressure 'HoneySweet' trees showed PPV symptoms and positive serological and molecular tests on some basal leaves only, and symptoms subsided during the growing season. ACLSV infection did not appear to affect PPV symptoms and PDV infection could not be detected in 'HoneySweet' throughout the course of the study despite graft inoculation. The evaluations of fruit quality of graft inoculated and un-inoculated 'HoneySweet' trees maintained nine years under the high and permanent infection pressure of PPV, ACLSV, and PDV confirmed not only the high resistance of 'HoneySweet' to PPV, but also suggested that 'HoneySweet' fruit maintain their quality and healthful properties when exposed not only to PPV but also to ACLSV and PDV. Clone C5, resistant to PPV, was deregulated as plum 'HoneySweet' in the U.S.A. in 2010. The meeting of U.S. International Research team for 'Honey Sweet' plum in Prague, May 2011 decided to submit an application for deregulation of plum cv. 'HoneySweet' in European Union. Members of International team are scientists form USA, France, Spain, Poland, Romania, Bulgaria and the Czech Republic. The Czech Republic is represented by Ass.Prof. Jaroslav Polák, DrSc. and Dr. Jiban Kumar from Crop Research Institute in Prague. International team under the leadership of Dr. Ralph Scorza, U.S.A. and Dr. Michel Ravelonandro, France is setting up the application for deregulation of 'HoneySweet' in EU. Crop Research Institute, Prague, and the Czech Republic are supposed to submit the application to EFSA. J. Polák is coordinating and setting up results of research on plum 'HoneySweet' in Europe. The growing of plum 'HoneySweet' in the Czech Republic will be principal contribution not only for fruit growers, but also for producers of plum brandy. It is unique opportunity to establish PPV free orchards and to grow high quality fruits.

a b

**Fig. 5. HoneySweet tree:**
a – with non-transgenic PPV infected bottom part. (Orig. J. Polák)
b – with cutted away non-transgenic PPV infected bottom part. (Orig. J. Polák)

The future is growing of high quality and productive crops resistant to plant diseases and pests, tolerant to herbicides, to ensure food security and to prevent hunger of people in the world. Genetic engineering will make possible to obtain high quality crop cultivars today very susceptible to diseases, highly resistant in the future, e.g. plum 'Domácí švestka' highly resistant to PPV.

1. Albrechtová L., Karešová R., Pluhař Z., Balcarová E., 1986. ELISA method used for the evaluation of resistance of plum cultivars to plum pox virus. Proc. 10th Conf. Plant Prot., Brno, 1986: 203-204.¨2. Clark M.F., Adams A.N., 1977. Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant virus. J. Gen. Virol. 34: 51-57. 3. Colwell R.K., Norse E.A., Pimentel D., Sharples F.E., Simberloff D., 1985. Genetic engineering in agriculture. Science 229: 111-114. 4. James C., 2011. Global status of commercialized Biotech/GM crops: 2011. ISAAA Brief No. 43. 5. Jarošová J., Kundu J.K., 2010. Simultaneous detection of stone fruit tree viruses by one-step multiplex RT-PCR. Scientia Horticulture 125: 68-72. 6. Malinowski, T., Zawadzka, B., Ravelonandro, M., Scorza, R., 1998. Preliminary report on the apparent breaking of resistance of a transgenic plum by chip-bud inoculation of Plum pox virus PPV-S. Acta Virol. 42: 241-243. 7. Malinowski, T., Cambra, M., Capote, N., Zawadzka, B., Gorris, M.T., Scorza, R., Ravelonandro, M., 2006. Field trials of plum clones transformed with the Plum pox viruscoat protein (PPV-CP) gene. Plant Dis. 90:1012-1018. 8. Polák J., Pívalová J., Jokeš M., Svoboda J., Scorza R., Ravelonandro M., 2005. Preliminary results of interactions of Plum pox virus (PPV), Prune dwarf virus (PDV), and Apple chlorotic leafspot virus (ACLSV) with transgenic plants of plum Prunus domestica, clone C5 grown in an open field. Phytopathol. Pol. 36: 115-122. 9. Polák J., Pívalová J., Kumar-Kundu J., Jokeš M., Scorza R., Ravelonandro M., 2008a. Behaviour of transgenic Plum pox virus-resistant Prunus domestica L. clone C5 grown in the open field under a high and permanent infection pressure of the PPV-Rec strain. J. Plant Pathol. 90 (Suppl. 1): S1.33-S1.36. 10. Polák J., Kumar-Kundu J., Pívalová J., Scorza R., Ravelonandro M., 2008b. Interactions of Plum pox virus strain Rec with Apple chlorotic leaf spot and Prune dwarf viruses in field growing transgenic plum Prunus domestica L., clone C. Plant Protect. Sci. 44: 1-5. 11. Ravelonandro M., Scorza R., Bachelier J.C., Labbone G., Lery L., Damsteegt V., Callahan A.M., Dunez J., 1997. Resistance of transgenic plums (Prunus domestica L.) to Plum pox virus infection. Plant Dis. 81: 1231-1235. 12. Ravelonandro M., Scorza R., Minoiu N., Zagrai I., Platon I., 2002. Field tests of transgenic plums in Romania. Plant's Health (Spec. ed.): 16-18. 13. Scorza R., Ravelonandro M., Callahan A.M., Cordts J.M., Fuchs M., Dunez J., Gonsalvez D., 1994. Transgenic plums (Prunus domestica L.) express the plum pox virus coat protein gene. Plant Cell. Rep. 14: 18-22. 14. Šubr Z., Pittnerová S., Glasa M., 2004. A simplified RT-PCR-based detection of recombinant Plum pox virus isolates. Acta Virologica 48: 173-176.

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**J. Polák**

## RECOVERING OF FRUIT CULTIVARS FROM PLANT VIRUSES AND PHYTOPLASMAS IN THE CZECH REPUBLIC: THE PAST, THE PRESENCE, AND THE FUTURE

***Ликвидация вирусов растений из растительного материала, особенно для экономически важных культур для Чехии, которые входят в систему сертификации, было основным заданием данных исследований. Представлены результаты оздоровления культур сливы, абрикоса и персика, пораженных вирусом шарки слив, а также культур яблонь, груши и черешни, инфицированных вирусами.***

***The task of research was to eliminate plant viruses from the basic plant material, especially from the Czech economically important cultivars included in the system of certification. Topical results of recovering plum, apricot, and peach cultivars infected with PPV, and apple, pear and sweet cherry cultivars infected with several economic important viruses are presented.***

Research of *in vitro* cultures of plants and the elimination of viruses in plants started in the Czechoslovakia fifty years ago (Svobodová J., 1962; 1964; 1965) in the Institute of Experimental Botany CZAS. First woody plants were recovered in 1972 (Svobodová-Santilliová J., 1972). Thermotherapy of fruit crops continued in the Research and Breeding Institute for Pomology (RBIP) in Holovousy (Janečková M. et al., 1978; Janečková M. and Blattný C. jun., 1980; Janečková et al., 1985, Janečková M., 1988). *Plum pox virus* (PPV) infected plum mother plants of maintenance breeding situated in the open field of Breeding Station Turnov, nord-east Bohemia in the eighties. Practically the all plum cultivars were PPV infected. Technical isolate for mother plants of stone fruits was established in the Research and Breeding Institute for Pomology (RBIP) in Holovousy still in the eighties. Some of especially Czech cultivars were recovered from PPV, and other economically important viruses (Fig.1). Janečková M. (1993) eliminated PPV, *Prunus necrotic ringspot virus* (PNRSV), and *Prune dwarf virus* (PDV) by thermotherapy using combination *in vivo* and *in vitro* cultures. Janečková (1995) applied thermotherapy *in vivo* with bud cultures *in vitro* to eliminate apple viruses. System of certification of virus free status of fruit trees in nurseries was established at the beginning of 21 century in the Czech Republic. Technical and space isolates for apple, pear, cherry and plum cultivars were situated to RBIP Holovousy, for apricot, peach, and almond cultivars to the Faculty of Horticulture (FH) Lednice, Mendel's University Brno. The third technical isolate was established in CRI Prague-Ruzyně. This isolate is divided into two parts, the first part for disease plants and collection of viruses for control purposes, the second one for healthy woody indicators and governmental reserve of basic plants of recovered cultivars.

**Fig.1: Fruit of peach cv. Fortuna, severe susceptible to PPV, severe rings and malformations. (Orig. J. Polák)**

The task of research was to eliminate plant viruses from the basic plant material, especially from the Czech economically important cultivars included in the system of certification. Basic plants of some foreign cultivars were obtained from abroad. Research projects with the aim to obtain virus and phytoplasma free cultivars of fruit trees are solved in the cooperation of Crop Research Institute (CRI) in Prague-Ruzyně, RBIP Holovousy, and FH Lednice. The first research project was established in 2004-2008, the second one continued in 2009-2011. The third research project has started this year for the period 2012-2016. The author of this contribution is the responsible person and co-ordinator of those projects. *In vivo* and *in vitro* thermotherapy was applied in the first research project. Thermotherapy, chemotherapy, and combination of thermotherapy with chemotherapy were exploited in the course of second research project. Topical results of recovering plum, apricot, and peach cultivars infected with PPV, and apple, pear and sweet cultivars infected with several economic important viruses are presented in this contribution.

**Material and Methods**

**Plant material, virus infection**

Candidate *in vivo* growing plants and/or *in vitro* cultures derived from plants of maintenance breeding were used for elimination of viruses and phytoplamas. Primary sources, cultivars of plum 'Bluefree', 'Domácí švestka', 'Čačanská lepotica'), apricot ('Velkopavlovická', 'Leskora', 'Hanita'), peach ('Redhaven', 'Earliglo'), sweet cherry ('Karešova', 'Rivan'), apple ('Idared', 'Šampion'), and pear ('Lucasova', 'Max Red Bartlett') were exploited in the first two research projects. Candidate plants were tested for the presence of viruses by ELISA Clark and Adams, 1977) and RT-PCR (Wetzel et al., 1991) before therapy, and viruses present in the plants were identified. Plum, apricot, and peach cultivars were infected with PPV (Fig.2). Apple cultivars were infected with *Apple chlorotic leafspot virus* (ACLSV), *Apple stem piting virus* (ASPV), and *Apple stem grooving virus* (ASGV). Pear cultivars were infected with ASPV, and cherry cultivars with PDV. Phytoplasmas were not detected in candidate plants.

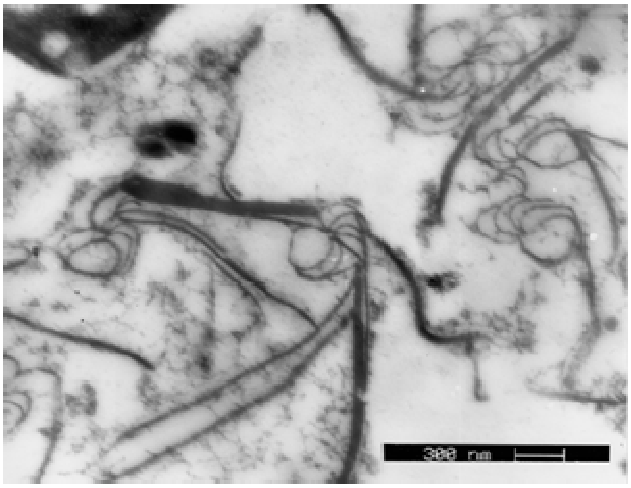

**Fig. 2. Pinwheels and laminated aggregates in cytoplasma of PPV infected leaf cell of plum (Orig. M. Jokeš)**

**Thermotherapy and Chemotherapy**

Thermotherapy *in vivo* plants was running for 5 to 22 days in a thermal room with controlled light and temperature setup at 37°C for stone fruits, and 39°C for pome fruits. The light setup was 14h day, and 10h night. 26 trees of plum (9), apricot (7), and peach (10) were taken for thermotherapy *in vivo* (Fig.3).

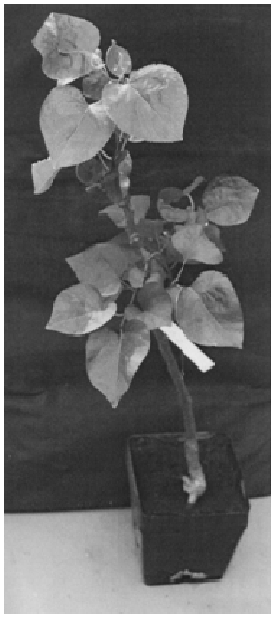

**Fig. 3. Apricot tree of cv. Velkopavlovická after the thermotherapy *in vivo* (Orig. A. Hauptmanová)**

For thermotherapy *in vitro* cultures actively growing shoot tips 5-10 mm in length were cut from shoots sprouting in laboratory conditions. The tips were cultured in Erlenmeyer flasks with Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) gelled with 0.7% Difco agar. The subcultures were prepared on fresh MS medium with the same composition in the period of 1 month. Cytokinin 6-benzylaminopurine (BAP) at concentration 1.5 $mg.l^{-1}$ was used in this medium for induction of multiplication. The heat treatment 34°C, 37°C, and 39°C was applied to the *in vitro* cultures on the same medium for 5-20 days.

For chemotherapy *in vitro* cultures were transferred onto fresh MS medium with ribavirin at concentration 5, 10, 20, and/or 40 $mg.l^{-1}$. Different periods of time from two to 27 weeks of ribavirin treatment were used in the individual trials. For each period at least 20 shoots were exposed to the one ribavirin concentration.

**Rooting of *in vitro* plants**

After the heat treatment or chemotherapy apical meristems with leaf primordia were transferred to a fresh multiplication MS medium. A period of about 6 months was necessary to obtain well established actively growing cultures. *In vitro* clones were tested by RT-PCR. Those *in vitro* clones that proved to be virus free were further multiplied androoted. For rooting of *in vitro* plants the different media, e.g. Paunovic et al. (2007) were applied. To confirm virus free status recovered *in vivo* trees were tested again by ELISA, and RT-PCR one year later.

Part of virus infected *in vitro* cultures was rooted without the heat treatment, and obtained *in vivo* trees were used for thermotherapy. Virus status of trees after the thermotherapy *in vivo* was tested by ELISA, and RT-PCR immediately and one year later.

**Results and Discussion**

The elimination of PPV was carried out in economically important plum, apricot and peach cultivars by *in vivo* thermotherapy, *in vitro* thermotherapy and chemotherapy in CRI Prague-Ruzyně. Four plants of plum cv. 'Čačanská lepotica' and one plant of cv. 'Domácí švestka' were PPV free after the finished *in vivo* thermotherapy, but only two trees of cv. 'Čačanská lepotica' remained PPV free nine months later. One plant of apricot cv. 'Leskora' and four plants of cv. 'Velkopavlovická' were PPV free after the finished *in vivo* thermotherapy. One tree of cv. 'Leskora' and one tree of cv. 'Velkopavlovická' remained PPV free nine months later. One plant of peach cv. 'Redhaven' was PPV free after the *in vivo* thermotherapy, but the presence of PPV was proved in this tree nine months later. None of the 10 peach trees of cvs. 'Redhaven'and 'Earliglo' was treated successfully. Results in detail of *in vivo* thermotherapy were published (Polák and Hauptmanová, 2009). The thermotherapy of *in vivo* trees was proved to be less efficient.

The thermotherapy, chemotherapy, and combination of thermotherapy with chemotherapy of *in vitro* cultures of plum cvs. 'Domácí švestka', 'Bluefree', and 'Čačanská lepotica', apricot cvs. 'Hanita' and 'Velkopavlovická', peach cvs. 'Redhaven'and 'Earliglo' were applied for elimination of PPV. The thermotherapy run at 34°C, and 37°C. The thermotherapy in 34°C was not enough for PPV elimination. *In vitro* cultures died during thermotherapy at 37°C. The combination of thermotherapy with chemotherapy of *in vitro cultures* of stone fruits was also not effective. The best results were obtained with chemotherapy by ribavirin in concentration 10 and 20 $mg.l^{-1}$. PPV was completely eliminated within 2-12 weeks of treatment. *In vitro* plants with bases of roots primordia were transferred into peat tablets. Plants rooted *in vivo* were transferred to greenhouse conditions. Recovered trees of plum cvs. 'Bluefree' (Fig.4), 'Domácí šveska', and 'Čačanská lepotica', and apricot cv. 'Hanita' were re-tested by ELISA and RT-PCR one year after the ribavirin treatment. The elimination of PPV was confirmed. Results in detail of elimination of PPV in plum cv. 'Bluefree' and in apricot cv. 'Hanita' were published (Hauptmanová and Polák, 2011). The chemotherapy of *in vitro* cultures was proved to be more efficient. Ribavirin in concentration 10 $mg.l^{-1}$ can be recommended for elimination of plant viruses from both stone and pome fruit species.

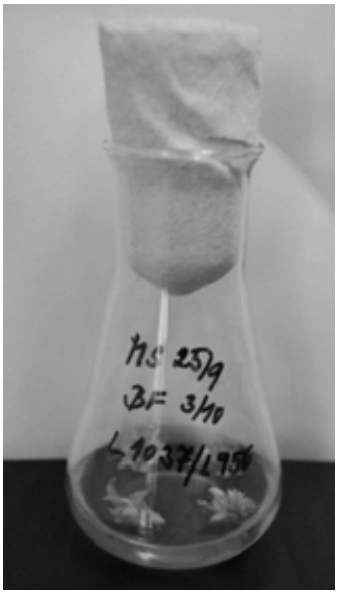

**Fig. 4. *In vitro* cultures of plum cv. Bluefree after the chemotherapy (Orig. A. Hauptmanová)**

The elimination of viruses was carried out in economically important apple, pear, and sweet cherry cultivars by *in vitro* thermotherapy and chemotherapy in RBIP Holovousy. The most virus free rooted plants (62) were obtained by *in vitro* chemotherapy. The only 13 virus free rooted plants in containers were obtained by *in vivo* thermotherapy. Recovered rooted trees were tested one year later. Eighteen pome fruit trees and three trees of sweet cherry remained virus free. Finally, virus free trees (at least 1 tree per cultivar) of two apple cultivars ('Idared', 'Šampion'), two pear

cultivars ('Lucasova', 'Max Red Bartlett'), and two sweet cherry cultivars ('Karešova', 'Rivan') were obtained. Results of *in vitro* chemotherapy and *in vivo* thermotherapy of *in vitro* cultures of apple, pear, and sweet cherry cultivars were published (Paprštein et al., 2007; 2008; 2011; Sedlák et al., 2007).

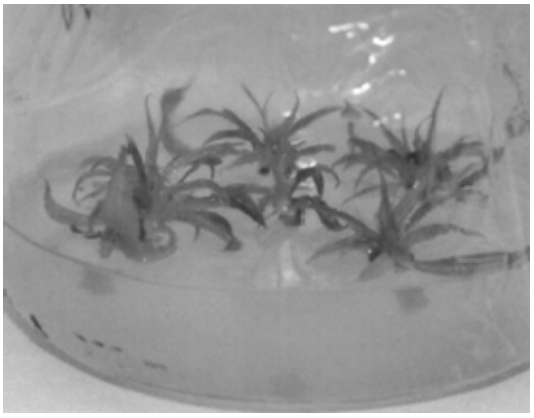

**Fig. 5. The cultivation of tips of peach cv. Redhaven after the therotherapy *in vivo* (Orig. B. Křižan)**

The elimination of viruses and phytoplasmas from commercial apricot and peach cultivars was carried out at HF Lednice. The same or similar methods and procedures as in CRI Prague and RBIP Holovousy were exploited. The best results were obtained with chemotherapy of *in vitro* cultures by ribavirin. Apricot cultivars 'Bergeron' and 'Velkopavlovická', peach cultivars 'Redhaven', 'Cresthaven' and 'Suncrest' were recovered with ribavirin in concentration 10 $mg.l^{-1}$. For recovering of apricot cv. 'Marlen' it was necessary to use ribavirin in concentration 20 $mg.l^{-1}$, or even 40 $mg.l^{-1}$ (Křižan et al., 2011). The elimination of viruses from stone fruit cultivars is more difficult than from pome fruit cultivars. Contributions important for our research of the elimination of viruses from stone fruit species were published in the past (Deogratias et al., 1989; Gabova, 1995; Knapp et al., 1995). Stone fruits, especially peaches are sensitive to the higher temperature, 36°C and more (Fig.5). Virus free trees of apple, pear, and sweet cherry cultivars will be included as the basic plants in the established system of certification. Some problems remain in chemotherapy of stone fruits, especially with peach cultivars. These problems and new chemotherapeutica will be investigated in new, the third research project.

1. Clark, M.F. and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. Journal of GeneralVirology,34: 475−483. 2. Deogratias, J.M., Dosba, F., Lutz, A., 1989. Eradication of prune dwarf virus, prunus necrotic ringspot virus, and apple chlorotic leaf spot virus in sweet cherries by a combination of chemotherapy, thermotherapy, and in vitro culture. Canadian Journal of Plant Pathology, 11: 337-342. 3. Gabova R.N., 1995. Chemotherapy treatment of Prunus spp. genotypes infected by plum pox virus. Plant Science (Sofia), 32: 16-18. 4. Hauptmanová A., Polák J., 2011. The elimination of Plum pox virus in plum cv. Bluefree and apricot cv. Hanita by chemotherapy of in vitro cultures. Hort. Sci. (Prague), 38: 49-53. 5. Howell W.E., Eastwell K.C., Li T.S.C., 2001. Heat treatment, chemo-therapy and hydroponic culture for obtaining virus-free trees of sweet cherry. Acta Horticulturae, 550: 455 – 457. 6. Janečková M., Zimandl B., Blattný C. jun., 1978. Effektivnost termoterapii virusnych podvojev jabloni. Učast. 3go Sovešč. Stran-členov SEV Tema 6.8., Sofia 1978: 32-35. (in Russia). 7. Janečková M., Blattný C. jun., 1980. Thermotherapeutic treatment of sour cherry ˇFanal' infected with green ring mottle. Sbor. ÚVTIZ – Ochr. Rostl. 16: 161-164. (in Czech). 8. Janečková M., Navrátil M., Pluhař Z., 1985. A rapid verification of health conditions of mother-graft stone trees after thermotherapy, maintained in natural isolate by ELISA. Sbor. ÚVTIZ – Ochr. Rostl. 21: 151-152. (in Czech). 9. Janečková M., 1988. The raspberry virus elimination programme in Czechoslovakia. Acta Horticult. 236: 45-50. 10. Janečková M., 1993. Elimination of virus complex (PPV, PNRSV, PDV) from plum varieties using combination of in vivo and in vitro cultures. Vědecké ovocnářské práce, 13: 51 – 64 (in Czech). 11. Janečková M., 1995. The elimination of apple viruses by combination of in vivothermotherapy with segmental budcultures in vitro. Vědecké ovocnářské práce, 14: 45-50. (in Czech). 12. Knapp E., Hanzer V., Weiss H., daCamara Machado A., Weiss B., Wang Q., Katinger H., Laimer da Camara Machado M., 1995. New aspects of virus elimination in fruit trees.Acta Horticulturae, 386: 409 – 418. 13. Křižan B., Ondrušiková E., Moudrá J., Eichmeier A., Hollainová V., 2011. Recovering of plants of apricot and peach cultivars from viruses by chemotherapy. Zahradnictví 12/2011: 18-19. 14. Murashige, T., and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497. 15. Paprštein F., Sedlák J., Zeman P., Svobodová L., Polák J., Hassan M., 2007. Preliminary results of in vitro chemotherapy of apple and pear. Inovation of Fruit Growing. RBIP Holovousy Ltd.: 29-35. ISBN 978-80-87030-03-5. (in Czech). 16. Paprštein f., Sedlák J., Polák J., Svobodová L., Hassan M., Bryxiová M., 2008. Results of in vitrothermotherapy of apple cultivars. Plant Cell, Tissue and Organ Culture, 3: 347-352. 17. Paprštein f., Sedlák J., Svobodová L., Polák J., Gadiou S., Zeman P., 2011. Elimination of viruses from fruit woody species and long term observation of health status. Vědecké práce ovocnářské, 22: 35-44. (in Czech). 18. Paunovic, S., Ruzic, D., Vujovic, T., Milenkovic, S., Jevremovic, D., 2007. In vitroproduction of plum pox virus – free plums by chemotherapy with ribavirin. Biotechnology and Biotechnik, 4: 417-421. 19. Polák J., Hauptmanová A., 2009. Preliminary results of in vivo thermotherapy of plum, apricot and peach cultivars artificially infected with PPV-M and PPV-D strains of Plum pox virus. Hort. Sci. (Prague) 36: 92-96. 20. Sedlák J., Paprštein f., Svobodová L., Polák J., Hassan M., Bryxiová M., 2011. Preliminary results of in vitrothermotherapy of apple and pear. Vědecké práce ovocnářské, 20: 73-80.(in Czech). 21. Svobodová J., 1962. Some notes on the behavior of virus Y in apical tissues of potato grown on agar medium. Plant Virology. Proc. 5th Conf. Czechoslovak Plantn virol., Prague 1962: 54-56. 22. Svobodová J., 1964. Cultivation of virus-free potato developed from meristem tissue cultures. O degeneraci brambor. Soubor Přednášek Mezinár. Konf. Východočeské nakladatelství Havlíčkův Brod, 1965: 123-127. (in Czech). 23. Svobodová J., 1965. Elimination of viruses by means of callus tissue culture. Viruses of Plants. Proc. Int. Conf. Plant Viruses, Wageningen, 1965: 48-53. 24. Svobodová-Santilliová J., 1972. Possibilities of elimination of poplar mosaic virus by means of poplar meristem and callus cultures method. Sbor. Věd. Prací IV. Celost. Konf. Ochr. Rostlin, Bratislava 1972, část 1: 81-90. (in Czech). 25. Wetzel, T., Candresse, T., Ravelonandro, M., Dunez, J. 1991. A polymerase chain reaction assay adapted to Plum pox potyvirus detection. Journal of Virological Methods, 33: 355–365.

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# VIRUSES OF FOREST TREES, VECTORS OF VIRAL TRANSMISSION AND METHODS FOR STUDYING PLANT VIRUSES

***В этой статье дается обзор литературы с акцентом на вирусы основных пород лесных деревьев, их векторы передачи и молекулярные методы, используемые для выявления, идентификации и изучения вирусов растений в лесных экосистемах. Были рассмотрены некоторые экспериментальные данные о вирусах, присутствующих в лесной почве, а также представлены точные данные о болезнетворных агентах некоторых видов деревьев в лесах Европы, США и других стран. В конце статьи была отмечена необходимость исследований вирусных патологических процессов в лесных насаждениях с целью предотвращения потерь в биоразнообразии лесных насаждений, снижения качеств древесины и т. д.***

***This article reviews the literature with focus on some of the major forest tree species viruses, vectors of viral transmission and molecular techniques used to identify, qualify and examine plant viruses in forest ecosystems. Some experimental data regarding soil inhabiting viruses was reviewed and particular information about causative agents of certain forest trees in Europe, USA and other countries was presented. The necessity of researches regarding more and more important role of viral pathological processes in forest ecosystems in order to prevent such issues as: loss of plants vigor, loss of wood quality etc. which were highlighted in the end of the article.***

**Introduction.** Forest decline has become a popular issue, especially in connection with air pollution emanating from industrialized areas, but such aspect as forest diseases, especially of virus etiology [49] have been considered of minor importance. There are not so many studies focused on plant viruses as infection agents of forest trees in particular, but many focused more on mixed types of diseases, incorporating fungi, mycoplasma and other infection activators rather than viruses. There were numbers of scrutinies regarding forested areas and nurseries and they seem to confirm the expansion of viruses in many plants with virus associated symptoms. Viruses induce alterations in a tree's metabolism and alter plants predisposition. Viruses reported in many forest ecosystems and recovered from forest tree species. Viruses are widespread pathogens in agricultural crops, weeds and forest trees in particular, as a result, they are considered to be important reservoirs of viruses [37]. Viruses can only be replicated in living cells, because they lack their own metabolic activities. Nowadays, plant pathologists and biologist commonly are not concerned with viruses of forest trees, instead focusing their attention on short-term agricultural and fruit crops, where virus impacts have been shown well in near-term outlook. In spite of this, viruses or virus-like particles (VLPs) of coniferous and deciduous forest tree species causing significant impact on plant communities [16]. They can be soil- and airborne, and are transmitted from one plant to the other by mechanical means through wounds, arthropods, nematodes or fungal vectors, and a considerable number by seed and pollen. The international transfer of contaminated plant material is another important factor of virus distribution. If the economic point of view is considered, we should be cognizant that virus diseased plants may increase production costs because of the possibly decreased growth of infected stock plants and that viruses may subsequently damage their market quality. Information based on nature of viruses and virus-like diseases in forest trees makes us scientists observant and conscious about the ways how to deal with these types of plant pathology-related problems but not as fully as we all probably would have liked it to be.

*Some specifics of forest trees viruses and their certain vectors.*

Unlike animal viruses, where hosts are mobile and often come into contact with each other, plant viruses need to cover the often large distances separating their fixed hosts. The mechanisms of virus-vector relationships often studied and reviews on the subject are published frequently [43]. Although the only well-characterized viruses yet detected in conifers have soil-inhabiting vectors [47], others with aerial vectors also play big role in infection transmission. A few polyphagous aphid species capable of transmitting numerous viruses have been recorded on conifers: Aphis fabae Scop, feeds and reproduces on species of Larix, Picea, Tsuga and Pinus Aulocorthum circumflexum (Buckton) on seedlings of Picea sitchensis Carr. [11]. If concerning viruses which are transmitted by organisms, which are well presented in soil, we can underline two main vectors: fungi and nematodes. Fungus-borne viruses can be split in two categories: (i) viruses belonging to the Tombusviridae family, which have isometric particles and are transmitted by Olpidium spp. and (ii) rod-shaped viruses mainly belonging to the Potyviridae or to an unassigned family that are transmitted by plasmodiophorids [45]. Parasitic fungi are coming in contact with their host plants under the form of motile zoospores, which can digest the root cell wall and penetrate into the cytoplasm, from where they will colonize the whole plant. Two different patterns of virus transmission exist. Some viruses, for instance in the genus Bymovirus, are present within the fungus cytoplasm early during formation of the future zoospores in infected plant cells. They will remain inside the zoospore until its cytoplasm is injected in the next host cell. In other cases, for instance in cucumber necrotic virus (genus Tombusvirus), the best-studied example of a fungus-transmitted virus, virions are specifically retained at the surface of the zoospore envelope, and inoculated into the plant upon cell wall digestion and fungal penetration. In this case, the receptors of the vector were partially characterized. They have been shown to be distributed at the surface of the zoospore of Olpidium bornovanus, and their chemical nature was identified as a glycoprotein. Some other viruses are transmitted from plant to plant by soil-inhabiting plant parasitic nematodes [52]. Known vector nematodes belong to the families Longidoridae (longidorids) and Trichodoridae (trichodorids) and transmit nepoviruses and tobraviruses, respectively. These nematodes are ectoparasites that remain outside the roots while feeding on epidermal cells located just behind the root tip. Transmission is a noncirculative process in which virus particles are retained at specific sites on the surface of the esophagus. Nematodes can transmit virus even after serial feeds on noninfected tissues and retain virus for periods of months. However, it was shown that transfer of infection from nematodes to trees can be prevented in some way, thus forest pathologists are able to reduce spread of nematode-transmitted viruses by using plant metabolites. Many plant constituents have been investigated for activity against plant-parasitic nematodes. The conditions under which compounds are effective against nematodes vary with the compounds [60]. These active compounds, or precursors of active compounds, can often be applied to soil as organic amendments, or refined and developed as biopesticide compounds.

To date, only a few comprehensive studies have attempted to detect viruses in forest soils and somehow classify them. One of them was done in forest soils in New York State [20]. Objectives of the survey were to evaluate elution and bait plant methods to detect infectious tobamoviruses. Soils were collected from two forest sites: Whiteface Mountain (WF) and Heiberg Forest (HF). The effectiveness of four buffers to elute tomato mosaic tobamovirus (ToMV) from organic and mineral fractions of WF soil amended with ToMV was tested, and virus content was assessed by enzyme-linked immunesorbent assay (ELISA). The effectiveness of Chenopodium quinoa (Willd.) bait plants to detect the virus also was tested. Both methods then were utilized to detect tobamoviruses in 11 WF and 2 HF soil samples. A phosphate buffer (100 mM, pH 7.0) eluted more ToMV from soil than the other buffers tested. Mineral soil bound more virus than organic soil. Virus recoveries from virus-amended organic and mineral soils were 3 and 10%, respectively, and the detection sensitivity was 10 to 20 ng/g of soil. Roots of bait plants grown in all virus-amended soils tested positive by ELISA, and virus concentrations averaged 10 ng/g. Both ToMV and tobacco mosaic tobamovirus (TMV) were transmitted to C. quinoa by elution from one of two HF soil samples but not from the WF soil samples. A tobamovirus was detected by bait planting in 12 of 73 (16%) root extracts representing 5 of 13 soil samples (38%). Tobamovirus-like particles were seen by transmission electron microscopy in 6 of 12 infected root extracts. After the experiment was done, it was stated that Tobamoviruses occur in forest soils in New York State. Abiotic soil transmission to trees may permit localized spread and persistence of these viruses in forest ecosystems. Another experiment was conducted in the German forests (10). Precedence was next: samples were collected from the area near the base of trees and seeds of some of the herbaceous virus indicator hosts were then planted in the soil which contained the samples and these plants were indexed for viruses. In the end of the experiment the majority of viruses were identified as Potex-, Tobamo-, Potyviruses (potato virus Y group), and TNV isolates. More than half the recovered viruses were potexviruses. PVSi was recovered twice directly from the roots of European beech (Fagus sylvatica), and from soil/root samples from beech, pine, oak, and spruce forests (30%, 30%, 20%, and 18%, respectively). Other viruses were detected as well. Tomato mosaic virus (ToMV) was detected in soil samples from pine, spruce, beech, and oak forests. It should be mentioned that there are always some factors which are significantly decrease quantity of viruses which could have been detected in soils. These are: (i) non-mechanically transmissible viruses were not surveyed, (ii) specific vectors (like nematods or/and fungi) might have not been present in all soil samples, (iii) the indicator plants might have not been sensitive to all the viruses presented in the samples, etc. In addition, there are many factors which determine the survival and spread of soilborne viruses. For example, soil qualities. Those may limit the spread of viruses with nematode vectors, so the survival of viruses in soil depends to a certain extent on their adsorption to clays. Adsorption may be affected by pH, with the consequence that low soil pH may prolong virus survival in soil in orested areas.

Many plant viruses have been also recovered from rivers and lakes, primarily in Central Europe [32]. Potex-, tombus-, tobamo-, and cucumoviruses (cucumber mosaic virus group) were detected along with ungrouped or as yet unidentified viruses [33]. The most important trees for forest ecosystems and forestry in particular include some of coniferous and deciduous trees so the main researches for identifying viruses specifically in these trees direct the need of observations. Some viruses of forest trees are listed below.

OAK. Yarwood & Hecht-Poinar [40] described a virus resembling TMV (Tobamovirus) in oak in California. Subsequently, the virus was detected in buds and young leaves of 11 symptomless species of Quercus and Lithocarpus [59]. The virus was transmitted from oak to herbaceous plants by conidia of the powdery mildew Sphaerotheca lanestris Harkn. [35]. In the mountains of the Rhineland, TMV was isolated from oaks displaying chlorotic flecking, mottling, and mosaic on deformed leaves. Viruses were observed in young oak seedlings after mechanical transmission [36]. TMV-like particles were detected in symptomatic oak in Germany, but mechanical transmission tests were unsuccessful. Similarly, Horvath and coworkers [29] detected TMV-like particles in malformed leaves of turkey oak (Q. cerris). A mosaic disease of blackjack oak (Q. marilandica) in the United States was graft-transmissible, but attempts at mechanical transmission were unsuccessful [6]. Other possible members of the group, such as potato mop-top virus, may be transmitted by root-infecting fungi (Plasmodiophorales: Spongospora subterranea Lagerh). Some virus-like symptoms such as distinct chlorotic lesions, ringspots and chlorotic mottle were observed on leaves of oak trees and seedlings (Quercus robur L) growing at several forest stands and nurseries in north Germany. Investigations by serological means demonstrated that the agent of virus-like symptoms of oak were not tobacco mosaic virus, tobacco necrosis virus, brome mosaic virus and cherry leafroll virus but were related to the cryptic virus group [9].

ASH. Virus-like diseases of European ash consisting of mosaic and leaf deformation have been described in Europe [8,39]. Viruses are widespread in the ash population affected by decline. Tobacco ringspot virus (TRSV), tobacco mosaic virus (TMV), and tomato ringspot virus (TmRSV) occur in ash in New York State [12]. The first two viruses were associated with foliar viruslike symptoms on ash in the field, but virus infection was not correlated with dieback. Virus particles resembling TMV, however, were detected in Quercus spp. and Acer spp. [22] in conditions that suggest that natural spread might have occurred with the aid of the powdery mildew fungus (Sphaerotheca lanestris Harkin). As for TmRSV, it first was identified in stump sprouts of a white ash that had declined was associated with foliar symptoms [19]. CLRV is another example of infection agent in trees [5]. Nienhaus and Hamacher transmitted a CLRV isolate to white ash (F. americana) seedlings that subsequently developed chlorotic spots, ringspots, and line patterns. Similar symptoms on European ash in the U.K. were associated with ArMV [17]. The trees became infected when growing in soil infested with viruliferous nematodes. Similar symptoms on European ash in the U.K. were associated with ArMV. The trees became infected when growing in soil infested with viruliferous nematodes. ArMV was mechanically transmitted to seedlings of European ash and flowering ash (F. ornus L.), which developed chlorotic local lesions, systemic chlorotic chevrons, and a chlorotic mottle, respectively.

BIRCH. Birch decline is a serious disease in northeastern USA and eastern Canada, affecting both white and yellow birch (Betula papyrifera and B.alleghaniensis). Most of the merchandable trees in the severely affected areas were killed in the 1935-55 period. Hansbrough [27] in 1953 transmitted a gold ringspot of white birch to seedlings but did not associate this virus with the decline. Later Berbee transmitted the line pattern of yellow birch to seedlings. The line pattern symptoms on both species consist of chlorotic lines forming oak-leaf designs, irregular rings or

linear flecks, sometimes accompanied by a mild mosaic. Until fully expanded, emerging leaves on infected trees generally are symptomless and some infected trees have a few, or no, foliar symptoms. These symptoms may be restricted to a few leaves on a few branches. The leaves remain on the trees until the end of the growing season. Chlorotic tissue turns nearly white during midsummer. The virus has been mechanically transmitted to Chenopodium, cowpea, cucumber, squash, and bean. Serological and host range studies demonstrate that this virus-causing line pattern of birch is a strain of apple mosaic. Finally, Berbee and Gotlieb concluded that apple mosaic virus (ApMV) was responsible for such disease flow in symptomatic birch [23]. It has been also reported that Cherry leaf roll virus, CLRV is widely distributed in birch trees. The abnormalities, caused by CLRV were revied by Hamacher and others. In CLRV-infected birch, the area occupied by vascular bundles was reduced in comparison to healthy trees. Phloem cells were partly collapsed or disorganized and cell walls were thickened. Meristematic cells were deformed and reduced in size or their development completely inhibited. Sclerenchyma and collenchyma developed earlier than normal and chloroplasts were malformed. Tannin accumulated in the epidermis, palisade, and spongy mesophyll cells of the leaf laminae. Parenchyma cells of petioles, leaf laminae, and veins became necrotic. Young roots of diseased trees showed collapsed cells in the pericycle and endodermis and an accumulation of phenolic compounds [25]. It was also shown that CLRV is widely distributed in B. pendula and B. pubescens throughout the Finnish forestry region [31]. Furthermore, dwarf birch, mountain birch, Kiilopaa birch and curly birch were confirmed to be previously unkown hosts of CLRV [55]. The main route of CLRV dispersal in birch in natural habitats is assumed to be pollen and seed transmission, which has been studied in detail before [15].

ELM. Recent investigations in forest, nurseries and public gardens have shown that viruses are widely spread in deciduous trees including elm trees (Ulmus sp.). Biological, serological and electron microscopic assays showed that viruses of elm trees such as Cherry leaf roll virus (CLRV), Elm mottle virus (EMoV), Arabis mosaic virus (ArMV) and Tobacco ringspot virus (TRSV) are present in some of the German parks and forests [3, 4]. Elm mottle virus of Ulmus minor was also reported to be found in Croatia [44]. In the United States, a graft-transmissible disease of American elm (U. americana L.) was reported in Ohio in 1927 [51].

MAPLE. Maple mosaic, maple line pattern or maple variegation virus reported from Europe, but apparently present in the northeastern United States also [2]. In 1980 it was reported that chlorotic spotting of the leaves was observed on several Acer saccharum seedlings, 2-3 years old, in Sainte-Anne-de-Bellevue, Canada. Symptoms induced on indicator plants inoculated with homogenate from affected leaves resembled those produced by tobacco mosaic virus. Concentrated preparations showed a UV spectrum like that of TMV and reacted with antiserum to an ash strain of the virus. Rod shaped particles were detected in leaf preparations of Acer and tobacco and in purified preparations [34].

BEECH. The most economically important deciduous tree for some of the European forest industries is European beech (Fagus sylvatica L.). Thereby, necessity to inspect viral diseases of this plant family seems to be obvious for economical and biodiversity reasons. In the U.K. and East Germany beech displaying chlorotic leaf mottling and spotting were reported [48]. TBRV was identified in symptomatic trees. Nienhaus and coworkers [38] isolated potex- and potyviruses from trees with similar symptoms in West Germany. One isolate was serologically identical with PYX, another with PVSi, and a third with bean yellow mosaic virus (BYMV) [58]. European beech trees infected with cherry leaf roll virus (CLRV) or brome mosaic virus (BMV) often exhibit irregular, meandering growth of branches and sometimes develop clawlike twigs with reduced internodes [26]. Single branches or twigs, particularly in the upper part of the canopy, may die, thus giving the tree a bristle appearance. The wood of virus-infected branches and twigs is brittle and dry. Leaf symptoms appear on single twigs or branches beginning during June. Young trees usually show more pronounced leaf symptoms than older trees. CLRV-infected leaves exhibit chlorotic line patterns, mosaic, or yellow stippling. Common leaf symptoms are small size, curling, and reduced growth of veins accompanied by chlorosis that becomes bright yellow.

CONIFERS. Viruses infect many conifer trees species in forest ecosystems [21]. Several coniferous species have been infected with viruses via the root system [28], where infection remained localized. There also have been reports of systemic virosis of conifers. Cech and coworkers [14] described an aphid-transmissible virosis of Norway spruce in Czech Republic. The disease was aphid- and graft-transmitted and rod-shaped particles were detected in needle and twig exudates in both symptomatic trees and inoculated seedlings. Biddle & Tinsley [7] observed VLPs in sap exudates of Sitka spruce with needle chlorosis and defoliation in the U.K. Rod-shaped VLPs also were observed in western white pine (P. monticola) and Scots pine. In 2006 a new virus was found in Pinus sylvestris L. in different pine populations in Germany and Hungary [13]. On the basis of sequence comparison with different RNA viruses and phylogenetic analysis in was assumed that virus proteins from pine show highest similarity to the homologous proteins of Beet cryptic virus 3 and of a cryptic virus of Pyrus pyrifolia. It should be mentioned that Cryptoviruses have not yet been reported to occur in Gymnosperms.There was also a report from USA, where scientists found tomato mosaic virus in red spruce trees on Whiteface Mountain, New York (54). By all above mentioned, we may conclude that viruses of conifers are widespread not only in Nothern and Central parts of Europe but also in transatlantic countries.

*Preffered methods for studying viral diseases of forest trees.*

Viruses of woody forest plants and of their seeds are known to be difficult to detect due to phenolic compounds in plant extracts and an often irregular distribution or the low concentration of the pathogens within the plants and seeds when using serological methods as a tool. But still these methods have to be regarded as important diagnostic tools when considering the restricted capacity of the test. The objectivity of electron microscopy has increased by the use of immunological reagents, but more up-to-date methods of detection are preferable for searching viruses in woody plants. The ELISA is one of the most sensitive immunological system. It was reported [18] that poplar mosaic virus was successfully detected in infected trees using ELISA method. Methods which detect nucleic acid are often essential when the pathogen being sought lack protein. A new perspective for the diagnosis gives the combination of grafting plants to transmit the pathogen to host plants and a modified technique of the polymerase chain reaction (PCR) as well as the hybridization technique to detect the assumed pathogen. The PCR and hybridization technique are sensitive methods to detect viruses of smallest amount. Werner et al. [57] evaluated a method for detecting cherry leaf roll virus (CLRV) in seeds of birch and concluded that PCR followed by immunocapture-reverse transcriptase is the most sensitive way to detect the viral RNAs without a radioactive detection, which makes the system cheaper

and more reliable for routine use. In comparison to ELISA techniques RT-PCR approaches represent a remarkable improvement in sensitivity [41]. The advantages of PCR as a diagnostic tool include exceptional sensitivity, speed, and versatility. PCR is generally 102 to 105 times more sensitive than enzyme-linked immunosorbent assay, the widely used serological diagnostic benchmark. Sensitivity is of particular importance when viruses occur at low concentration (dormant plant tissues, woody tissue) or are unevenly distributed. Applications of PCR-based plant virus diagnosis usually include the following:

1. Germplasm screening;
2. Field surveys to determine virus incidence and geographic distribution;
3. Provision of virus-free planting material;
4. Domestic and international plant quarantine;
5. Detection of mixed virus infections;
6. Analysis of virus distribution in different plant tissues;
7. Identification of alternative host plants;
8. Evaluation of virus-resistant or -tolerant cultivars;
9. Analysis of virus transmission by insect, nematode, or fungal vectors.

In the early 1990s, the newest method of DNA amplification, the polymerase chain reaction (PCR), was introduced for plant pathogen detection. It provides a method of exponentially amplifying specific DNA sequences by in vitro DNA synthesis. Depending on the specificity of the primers, the amplification products can provide both narrow and broad detection capabilities for various isolates of a pathogen. For the application of viral RNA sequences to PCR, cDNA is synthesized by reverse transcription (RT) and amplified by PCR (RT-PCR). With the availability of nucleotide sequences for many plant viruses and their strains, the development of RT-PCR assays for the detection and diagnosis of viruses in plant tissues and vectors has become feasible [50]. PCR methodology has been extensively applied to detect viroids, viruses, bacteria, mycoplasma-like organisms, fungi, and nematodes infecting various plant species [24]. This has opened new avenues for epidemiological studies such as for analysis of molecular virus-vector interactions and virus localization in the vector and it may enable the development of novel approaches for the control of virus spread by vectors. Successful virus detection of tobra- and nepoviruses has been reported in viruliferous soil-inhabiting nematodes [53]. RT-PCR for the detection of viruses is severalfold more sensitive than ELISA. Whereas ELISA detects virus concentrations in the lower nanograms or picograms, RT-PCR is capable of detecting viral nucleic acids in femtograms (fg). Thus, the prospect of detecting plant viruses by RT-PCR has increased, especially those which occur in very low concentrations in their vectors, e.g., nonpersistently transmissible viruses.

Inhibitors of RT or PCR can be effectively eliminated by capture of virus particles (Virus-Capture PCR) from crude plant tissue or vector extracts by the surface of polypropylene PCR tubes or microtiter plates or by polystyrene ELISA plates.Components of crude plant extracts that would otherwise inhibit RT-PCR are washed away. Immunocapture PCR appears to be the method of choice when specific antisera are available and highest sensitivity is required [30, 46], e.g., for certification of virus-free planting material. Immunocapture of serologically diverse virus isolates or multiple strains/species requires broad-spectrum antisera or a mixture of different antibodies. Immunocapture PCR or RT-PCR works reliably for the entire spectrum of plant viruses, including enveloped tospoviruses [56].

These biological and molecular techniques have given a large tool kit for the detection and diagnosis of plant viruses, thus researches may be done in various ways. But if one wishes to determine whether a plant is virus infected, say for quarantine purposes, one does not necessarily need a sophisticated technique that identifies a virus strain. On the other hand, if one is studying the durability of a potential resistance gene (or transgene) it is very useful to have an understanding of the range of variation of the virus. Thus, one has to select the best technique for what is wanted.

**Conclusion.** Recent researches found that viruses are predisposing factors leading to early senescence of trees. Senescence reduces the regeneration capacity of the host plants, and the juvenile metabolic vigor is lost. Viruses predispose trees to other damaging factors and lead to premature senescence. Under abiotic stress conditions the infected trees have less potential for recovery from inciting factors than non-infected trees. All these events may and should be controlled by measures, which prevent or at least decrease extent of impact of diseases caused by or/and associated with viruses. When decisions are made over which control measures (biological, chemical, phytosanitary, etc.) to deploy and whether to use a measure alone or together with others, the implementation of chosen methods should be done with full responsibility. Some virus control measures are generic, while others are so specific that they only apply to particular pathosystems in certain agro-ecological situations. In fact, to be adopted control measures also need to be ecologically sustainable, robust, affordable and compatible with standard agricultural practices. For example, a particular type of control measure may be unsuitable for environmental or socio-economic reasons as chemical control may cause build up of toxic residues that are harmful to mankind, domestic animals and wildlife or there is unforeseen accumulation of damaging pests or other pathogens and its use is prohibited entirely in true 'organic'production systems (42). If nurseries are considered, elimination of virus-infected nursery stock can prevent the introduction of viruses into new areas. Vector control should be considered in nurseries. For sure, If we chose plants from nurseries without testing them on presence or propagation of any kind of viruses or virus potentially-related vectors it might later expand into infection over a large area (for example, public gardens, national reserves, etc.). The spread of viruses in seed can be prevented by appropriate indexing programs and the production of seed and propagating material from virus-free trees.

Virus infections in forest plants pose a worldwide challenge to achieving satisfactory yields and quality of produce. But nowadays, the role of viruses which affect and persist in forest trees and presented in forest soils is neglected. As were mentioned in this article, many viruses of forests haven't been identified yet or their influence on the flow of some associated with viruses diseases haven't been studied properly. Thus, we can not estimate correctly what are the main causes of diseases – whether they caused by microbiological agents or by virological agents. Consequently, the role of viruses may be far underesteemated for forest biodiversity, forest protection services and forestry-based industries. Knowledge of viral processes is necessary for the nature of plant ecosystems and this basic concept illustrates our vision very well – the more we know about viruses the more we can affect them and regulate their pathogenesis. Hopefully, an increasingly sophisticated and diverse range of molecular control methods are becoming available to meet the challenge for virological detection and identification. For example, the development of molecular techniques reveals the characterization of DNA or RNA virus genomes and their properties. From the taxonomic viewpoint, this has led to a large increase in the number of plant virus species and genera that have been distinguished and also to the establishment of quantitative

criteria to delimit different species [1]. Concerning plant pathology, these methods have given a huge toolbox for scientists for fast and accurate work with yet unknown infection agents. In conclussion, maintaining the vigor of our forests by using modern techniques and practices for identification, control and prevention of virus-related processes in trees and soils should be main priority for modern day plant pathologists across the globe.

1. Adams M.J., Antoniw J.F., Bar-Joseph M., Brunt A.A. and others. The new plant virus family Flexiviridae and assessment of molecular criteria for species demarcation // Arch. of Virol. – 2004. – Vol. 149. P. 1045–1060. 2. Agrios G.N. Virus and mycoplasma diseases of shade and ornamental trees // Journal of Arboriculture. – 1975. – Vol. 1(3). p. 41-47. 3. Bandte M., Eisold A.M., Lukacs N., Buttner C. Virological investigations on diseased European White Elm (Ulmus leavis) // Julius-Kühn-Archiv. – 2010. – Vol. 428. – P. 370. 4. Bandte M., Essing M., Obermeier C., Buttner C. Virus-diseased Ulmus laevis in Eastern Germany // Invest Agrar: Sist. Recur. For. – 2004. – Vol. 13(1). – P. 65-72. 5. Bandte M., Buttner C. Occurence, transmission and diagnosis of cherry leafroll nepovirus – a literature review // Pflanzenschutzberichte. – 2001. – Vol. 59. – P. 1-19. 6. Barnett O.W. Mosaic of blackjack oak // Plant Dis. Rep. – 1971. – Vol. 55. – P. 411. 7. Biddle P.G., Tinsley T.W. Virus diseases of conifers in Great Britain // Nature. – 1968. – Vol. 219. – P. 1387-1388. 8. Bojnansky V. Ash (Fraxinus excelsior) granular mosaic // Pro Ustavu Exp. Fytopathol. Entomol. –1976. – Vol. 1976. – P. 41-51. 9. Buttner C., Fuhrling M. Studies on virus infection of diseased Quercus robur (L) from forest stands in northern Germany // Annales des Sciences Forestieres. – 1996. – Vol. 53 (2-3). – P. 383-388. 10. Buttner C., Nienhaus F. Virus contamination of soils in forest ecosystems of the Federal Republic of Germany // Eur. J. For. Pathol. – 1989. – Vol. 19. – P. 47-53. 11. Carter C.I., Eastop V.F. Mindarus obliquus (Chol.) (Homoptera, Aphidoidea) new to Britain and records of two other aphids recently found feeding on conifers // Entomologist's mon. Mag. – 1973. – Vol. 108. – P. 202-204. 12. Castello J.D., Amico L.A., O'Shea M.T. Detection of tobacco mosaic and tobacco ringspot viruses in white ash trees by enzyme-linked immunosorbent assay // Plant Dis. – I984. – Vol. 68. – P. 787-790. 13. Castello J.D., Wargo P.M., Jacobi V., Bachand G.D. and others. Tomato mosaic virus infection of red spruce on Whiteface Mountain, New York: prevalence and potential impact // Can. J. For. Res. – 1995. – Vol. 25. – P. 1340-1345. 14. Cech M., Kralik O., Blattny C. Rod-shaped particles associated with virosis of spruce // Phytopathology. – 1961. – Vol. 51. – P. 183-85. 15. Cooper J.I. The possible epidemiological significance of pollen and seed transmission in the cherry leaf roll virus/Betula spp., complex // Mitteilungen der Biologischen Bundesanstalt fur Land- und Forstwirtschaft, Berlin-Dahlem. – 1976. – Vol. 170. – P. 17–27. 16. Cooper J.I., Jones R.A.C. Wild plants and viruses: under-investigated ecosystems // Advances in Virus Research. – 2006. – Vol. 67. – P. 1-47.17. Cooper J.I., Sweet J.B. The detection of viruses with nematode vectors in six woody hosts // Forestry. – 1976. – Vol. 49. P. 73-78. 18. Cooper J.I., Edwards M.L. The distribution of poplar mosaic virus in hybrid poplars and virus detection by ELISA // Annals of Applied Biology. – 1981. – Vol. 99(1). – P. 53–61. 19. Ferris M. A., Castello J. D. Detection of tomato ringspot virus in white ash and adjacent vegetation in Central New York // Can. J. For. Res. – 1988. – Vol. 18. – P. 813-817. 20. Fillhart R.C., Bachand G.D., Castello J.D. Detection of Infectious Tobamoviruses in Forest Soils // Appl. Environ. Microbiol. – 1998. – Vol. 64 (4). – P. 1430–1435. 21. Flachmann M., Lesemann D.E., Frenzel. B., Koenig R. Isometric virus-like particles in Abies alba Mill, and other Abies species: Partial purification and improved detection by means of immunoelectron microscopy // J. Phytopathol. – 1990. – Vol. 129. – P. 193-202. 22. Fuhrling M., Buttner C. Detection of tobamoviruses in sycamore maple (Acer Pseudoplatanus L.) exhibiting mottling and leaf deformation // Forstwissenschaftliches Centralblatt. – 1998. – Vol. 117(2). – P. 92-97. 23. Gotlieb A.R., Berbee J.G. Line pattern of birch caused by apple mosaic virus // Phytopathology. – 1973. – Vol. 63. – P. 1470-1477. 24. Hadidi A., Levy L., Podleckis E.V. Polymerase chain reaction technology in plant pathology // Molecular Methods in Plant Pathology. – Boca raton, FL, 1995. 25. Hamacher J., Kreutz G., Jacob S. Histoiogische Untersuchungen an Kirschenblattrollvirus-infizierten Birken // Meded. Rijksfac. Landbouwwet. Gent. – 1987. – Vol. 52(3a). – P. 1049-1057. 26. Hamacher J., Quadt A. Isolation of cherry leaf roll and brome mosaic viruses from European beech and transmission to beech seedlings // Plant Dis. – 1994. – Vol. 78. – P. 849-853. 27. Hansbrough J.R. The significance of the fungi and viruses associated with the birch decline: report of the symposium on birch decline, Ottawa, 1953. – Can. Dept. of Agr. Forest Div., Ottawa. 1953. 28. Harrison B.D. Infection of gymnosperms with nematode transmitted viruses of flowering plants // Virology. – 1964. – Vol. 24. – P. 228-229. 29. Horvath J., Eke I., Gal T., Dezsery M. Demonstration of virus-like particles in sweet chestnut and oak with leaf deformations in Hungary // Z. Pjlanzenkr. Pjlanzenschutz. – 1975. – Vol. 82. – P. 498-502. 30. Jacobi V., Bachand G.D., Hamelin R.C., Castello J.D. Development of a multiplex immunocapture RT-PCR assay for detection of tomato and tobacco mosaic tobamoviruses // Journal of Virological Methods. – 1998. – Vol. 74. – P. 167-178. 31. Jalkanen R., Buttner C., von Bargen S. Cherry leaf roll virus abundant on Betula pubescens in Finland // Silva Fennica. – 2007. – Vol. 41(4). P. 755–762. 32. Koenig R. Plant viruses in rivers and lakes // Adv. Virus Res. – 1986. – Vol. 31. – P. 321-333. 33. Koenig R., Lesemann D.E. Plant viruses in German rivers and lakes. I. Tombusviruses. a potexvirus and carnation mottle virus // Phytopathol. Z. – 1985. – Vol. 112. – P. 105-116. 34. Lana A.F., Thomas O.T., Peterson J.F. A virus isolated from sugar maple // Phytopathologische Zeitschrift. – 1980. – Vol. 97(3). – P. 214-218. 5. Nienhaus F. Tobacco mosaic virus strains extracted from conidia of powdery mildews // Virology. – 1971. – Vol. 46. – P. 504-505. 36. Nienhaus F. Viren und virusverdachtige Erkrankungen in Eichen (Quercus robur und Quercus sessiliflora) // Z. Pjlanzenkr. Pjlanzenschutz. – 1975. – Vol. 82. – P. 739-749. 37. Nienhaus F. Infectious diseases in forest trees caused by viruses, mycoplasma-like organisms and primitive bacteria // Experientia. – 1985. – Vol. 41. – P. 597-603. 38. Nienhaus F., Ebrahim-Nesbat F., Fricke M., Buttner C., Welter K. Investigations on viruses from declining beech trees in Rhineland and Westfalia, Federal Republic of Germany // Eur. J. For. Pathol. –1985. – Vol. 15. – P. 402-411. 39. Nienhaus F., Hamacher J. Virosen in Eschen // Allg. Forst. Z. – 1989. – Vol. 45. – P. 385-386. 40. Nienhaus F., Yarwood C.E. Transmissiun of virus from oak leaves fractionated with Sephadex // Phytopathology. – 1972. – Vol. 62. – Vol. 313-315. 41. Nolasco G., de Blas C., Torres V., Ponz F. A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens // Journal of Virological Methods. – 1993. – Vol. 45. – P. 201-218. 42. Perring T.M., Gruenhagen N.M., Farrar C.A. Management of plant viral diseases through chemical control of insect vectors // Annu. Rev. Entomol. – 1999. – Vol. 44. – P. 457–481. 43. Pirone T.P., Perry K.L. Aphids: non-persistent transmission // Advances in botanical research. – 2002. – Vol. 36. – P. 1-19. 44. Plese N., Juretic N. Virus disease of field elm (Ulmus minor Mill.) in Croatia // Šumarski List. – 1999. – Vol. 123(3). – P. 95-100. 45. Rochon D., Kakani K., Robbins M., Reade R. Molecular aspects of plant virus transmission by olpidium and plasmodiophorid vectors // Annu. Rev. Phytopathol. – 2004 – Vol. 42. – P. 211–241. 46. Rowhani A., Maningas M.A., Lile L.S., Daubert S.D., Golino D.A. Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions // Phytopathology. – 1995. – Vol. 85. – P. 347-352. 47. Ruehle J. L. Nematodes and Forest Trees – Types of Damage to Tree Roots // Annual Review of Phytopathology. – 1973. – Vol. 11. – P. 99-118. 48. Schmelzer K., Schmidt H.E., Schmidt H.B. Viruskrankheiten und virusverdachtige Erscheinungen an Forstgeholzen // Arch. Forstwes. – 1966. – Vol. 15. – P. 107-120. 49. Schutt P., Cowling E. B. Waldsterben, a general decline of forests in Central Europe: Symptoms, development, and possible causes // Plant Dis. – 1985. – Vol. 69. – P. 548-558. 50. Singh R.P. Reverse-transcription polymerase chain reaction for the detection of viruses from plants and aphids // Journal of Virological Methods. – 1998. – Vol. 74. – P. 125-138. 51. Swingle R.U., Tilford P.E., Irish C.F. A graft transmissible mosaic of American elm // Phytopathology. – 1943. – Vol. 33. – P. 1196-1200. 52. Taylor C.E., Brown D.J.F. (1997). Nematode vectors of plant viruses. (Wallingford,UK: CAB International), p. 286. 53. Van der Wilk F., Korsman M., Zoon F. Detection of tobacco rattle virus in nematodes by reverse transcription and polymerase chain reaction // European Journal of Plant Pathology. – 1994. – Vol. 100. – P. 109-112. 54. Veliceasa D., Enunlu N., Kos P.B, Koster S. and others. Searching for a new putative cryptic virus in Pinus sylvestris L. // Virus Genes. – 2006. – Vol. 32(2). – P. 177-186. 55. von Bargen S., Grubits E., Jalkanen R., Büttner C. Cherry leaf roll virus – an emerging virus in Finland? // Silva Fennica. – 2009. – Vol. 43(5). – P. 727-738. 56. Weekes R., Baker I., Wood K.R. An RT-PCR test for the detection of tomato spotted wilt tospovirus incorporating immunocapture and colorimetric estimation // Journal of Phytopathology/ – 1996. – Vol. 144. – P. 575-580. 57. Werner R., Muhlbach H.-P., Buttner C. Detection of cherry leafroll nepovirus (CLRV) in birch, beech and petunia by immunocapture-RT-PCR using a conserved primer pair // Eur. J. For. Path. – 1997. – Vol. 27. – P. 309-318. 58. Winter S., Nienhaus F. Identification of viruses from European beech (Fagus sylvatica L.) of declining forests in Northrhine-Westfalia (FRG) // Eur. J. For. Pathol. – 1989.¬ – Vol. 19. – P. 111-118. 59. Yarwood C.E., Hecht-Poinar E. A virus resembling tobacco mosaic virus in oak // Phytopathology. – 1970. – Vol. 60. – P. 1320 (Abstr.). 60. Zasada I.A., Ferris H. Nematode suppression with brassicaceous amendments: application based upon glucosinolate profiles // Soil Biology and Biochemistry. – 2004. – Vol. 36. – P. 1017-1024.

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# VIRAL INFECTIONS OF REPTILES: A REAL THREATFOR HEALTH OF HUMANS AND WARM-BLOODED ANIMALS

***Статья проясняет необходимость обратить внимание на вопрос распротранения вирусов среди рептилий, которые могут представлять серьезную угрозу для здоровья людей и животных. Проведены пилотные исследования по получению чувствительной системы для детекции вирусов рептилий, которые в дальнейшем будут использованы для рарзработки диагностических методов.***

***This paper deals with problems concerning the spreading of reptilian viruses which may be a serious threat for human and animal health. Preliminary studies have been carried out aiming the elaboration of a sensible test system permitting to detect reptilian viruses and to develop diagnostics methods.***

**Introduction.** Viral diseases of poikilotherm animals as well as viral agents transmitted or/and persisted in poikilotherm reservoir animals are now an almost unstudied and actual problem of contemporary virology. Investigations of reptilian pathology being today carried out belong to relatively new scientific trends which have become to develop rapidly during these last years [2, 13]. Research of disease agents able to infect both cold- and warm-blooded hosts is mostly focused on reptiles participation in epidemiology of diseases caused by viruses belonging to families mentioned above. It should also emphasize the importance of reptile viral disease research for veterinary specialists dealing with animal patients kept at home, the studies in this field being also essential from the scientific point of view concerning taxonomic investigations and virus evolution. In Ukraine there are almost no investigations of viral diseases whose victims and/or vectors are reptiles.

**Object of this work** is to draw attention to the importance of reptile viral infection problem as a possible source of virus spreading among animals and humans. In addition, the aim of our experimental research is to elaborate some protocols permitting to obtain virus susceptible cell systems adequate for isolation of reptile viruses.

Today representatives of all *Reptilia* classes are known to be possible intermediate hosts or reservoirs of different pathogens [2, 4, 5, 13]. Numerous viruses isolated from these animals have been described including those belonging to the arbovirus group (togaviruses, flaviviruses, rhabdoviruses, and bunyaviruses) transmitted by arthropods and causing infections of humans as well as of other mammals and birds [4,6,10]. Numerous studies prove arboviruses to possess enormous pathogenic possibilities. During last two-three decades, a lot of jointed demographic, socio-economical, and ecological factors led to a unique combination of previously absent conditions being especially favorable for increasing of epidemic potencies for numerous arboviruses (yellow fever, West Nile, dengue fever, and Chikungunya viruses) as well as for their spreading on territories where they had been previously absent, such spreading being accompanied by devastating epidemics [3,4]. The scientific data have already proved different members of the arbovirus group to be able to infect any reptilian species [26]. Under certain temperature conditions, viremia can appear in these animals. The recent studies demonstrate some arboviruses are able to persist in reptilian organisms during the winter period. Such a factor plays an outstanding role in infective epidemiology of diseases caused by these viruses. The West Nile virus (WNV) causes an arboviral disease endemic for Africa, Asia Europe, Europe, and Oceania, the infection being transmitted by mosquitoes of the *Culicidae* family. This virus infects susceptible mammalian species including humans as well as avian species causing the development of meningoencephalitis. The first epizootic outbreak due to this virus activity among birds was registered in the USA (New-York) in 1999 [12]. Before 2002 the virus spread on territories of all states; 120 humans fell ill in the same years, 11 of them died [2]. In 2001 first cases of alligator disease were reported from the state of Florida alligator farms; next year such reports were from farms of Georgia as well as from a Nile crocodile farm in Israel and from Mexico where the disease had been detected among wild crocodiles [2,10]. Until 2005, the disease was registered in states Texas, Louisiana, and Idaho. In Louisiana, 5000 young alligators perished, four cases of human infection among the farm workers have been found [30]. Seropositive individuals were detected in epizootic nidi among wild alligators [2]. The West Nile virus is a typical emergent infection for *Crocodylidae;* it is probably due to introduction of a viral strain being pathogenic for humans, birds, and horses. There are some data proving a high viremia degree in diseased alligators associated with marked virus secretion. Taking into consideration the peculiarities of epizootic outbreaks it is foreseen the infective agent to be spread among sensible *Crocodylidae* species by other ways, not only by transmission. It has been recently reported about parenteral and oral virus inoculation to alligators [13]. These data suggest a possible danger of infection for humans being in direct contact with animal feces and tissues [2,14].

The Japanese encephalitis virus (JEV) has been isolated from Chinese rat snakes (Elaphe rufodorsata) in Korea [15]. And the antibodies to JEV has been found in cobras (Naja naja) in Hong Kong. Transmission studies with Japanese encephalitis virus have shown that lizards can be infected with this virus both by parenteral inoculation and, in some species, by feeding on infected mosquitoes. Infected animals develop viremia, and the development of viremia is temperature dependent [205]. No clinical symptoms were reported in the lizards in that study. Direct virus detection in naturally infected reptiles by isolation in cell culture or by RT-PCR has been described less frequently[13].

Most alphaviruses can infect a wide range of vertebrates, mostly birds and mammals,but several have also been reported in reptiles. Studies on alphaviruses in reptiles have mostly focused on the possible role of these animals for the transmission of alphaviruses to humans and livestock. This has led to a focus on persistence of alphaviruses in reptiles, particularly viral persistence over winter in temperate regions in the absence of mosquito activity. Evidence of infections with eastern equine encephalitis (EEE) virus and western equine encephalitis (WEE) virus, either by isolation or serology, have been reported in a variety of chelonians, lizards (including members of the families Lacertidae, Teiidae, Iguanidae, Agamidae, and Gekkonidae) and snakes (including members of the families Colubridae, Elapidae, and Crotalidae)[10]. Transmission studies have been performed in garter snakes to determine whether the virus can overwinter in snakes, can result in antibody production, and be infectious to mosquitoes. Environmental (body) temperature affects viremia, with no viremia detected in experimentally infected snakes during torpor, and a lag time of several days required to detect the virus after an animal emerges and is

warmed. In some experimental studies, a persistent viremia was seen.[10] A transmission study involving subcutaneous injection of spotted turtles (Clemmys guttata) with EEE virus led to the development of viremia and neutralizing antibodies in infected turtles. Subcutaneous infection of Texas tortoises (Gopherus berlandieri) with WEE virus led to high titer viremia over an extended period of time. Viremia was longer at lower temperatures than at high environmental temperatures. None of the transmission studies described any signs of clinical disease in infected reptiles [13].

The investigations carried out by scientists of several universities in Alabama prove the poikilotherm vertebrates may be reservoir hosts of the Eastern equine encephalomyelitis virus (EEEV) [11]. During 2001-2003, females of blood-suckling *Culicidae* mosquitoes belonging to *Culex, Aedes, Culiseta,* and *Uranotaenia* genera were tested as possible EEEV-transmitting vectors. The data obtained show that certain mosquito species of genera *Uranotaenia* and *Culex (U. sapphirina, C. erraticus,* and *C.peccator)* collected during the transmission period (from April to October) prefer poikilotherm vertebrates as their hosts. The species *C.peccator* belongs to the subgenus *Melanoconion,* its members being vectors of alphaviruses [6,11]. During the study period the EEEV was detected in all mosquito species. Poikilotherm animal species were supposed to be possible EEEV reservoirs in South-Eastern territories of the USA, mosquito species *C. peccator* and *U.sapphirina* being found to feed on reptiles and amphibians. There are also publications concerning the EEEV detection in other mosquito species – *Aedes vexans* and *Culiseta melanura* feeding also on reptiles and amphibians; they may be an important link in the chain of the EEEV spreading. According to the data obtained by P.Ya.Kilochyts'ki[8], N.P.Kilochyts'ka, and V.P.Sheremet, the urbanization process has given a stimulus for a new direction of mosquito evolution accompanied by intensive adaptation for existence in new environment conditions. Seven mosquito species including *Aedes vexans* and other members of *Aedes* and *Culex* genera are now considered as potentially dangerous vectors of human and animal infective agents [3]. The mosquito genera being the most important for arbovirus transmission are *Aedes, Culex,* and *Haemagogus* [3,6].

According to the data of official Interpol and CITES statistics, the world volume of illegal market turnover concerning endangered animal species reaches from $8-10 up to $20 milliard annually; it is the second position comparing to drug market gains (forestalling the arm trade ones). [1,16] (table 1).

***Table* 1. Volume of the alive reptiles market turnover (for some countries)**

| Country | Period of time, years | Quantity of alive reptiles imported (annually) | Quantity of alive reptiles exported |
|---|---|---|---|
| USA | 1989-1997 | 2,000,000 | 9,000,000 * |
| | 2002 | 2,250,000 | |
| EC countries | 1992-2002 | 11,000** | |
| South Korea | 1993 | 1,400,000*** | |
| Italy | 1993 | 1,300,000 | |
| Ukraine | 1999-2009 | 1,000,000 | |

*In most cases they are read-eared turtles *(Trachemys scripta elegans);* according to the USA legislation, any species of fresh water turtles cannot be imported, the trade within the country being forbidden if their test diameter exceeds 10 cm; it is a preventive measure against salmonellosis outbreaks, these animals being their common source.

**These data concern only crocodiles and monitors *(Varanus* sp.).

***These data concern only read-eared turtles.

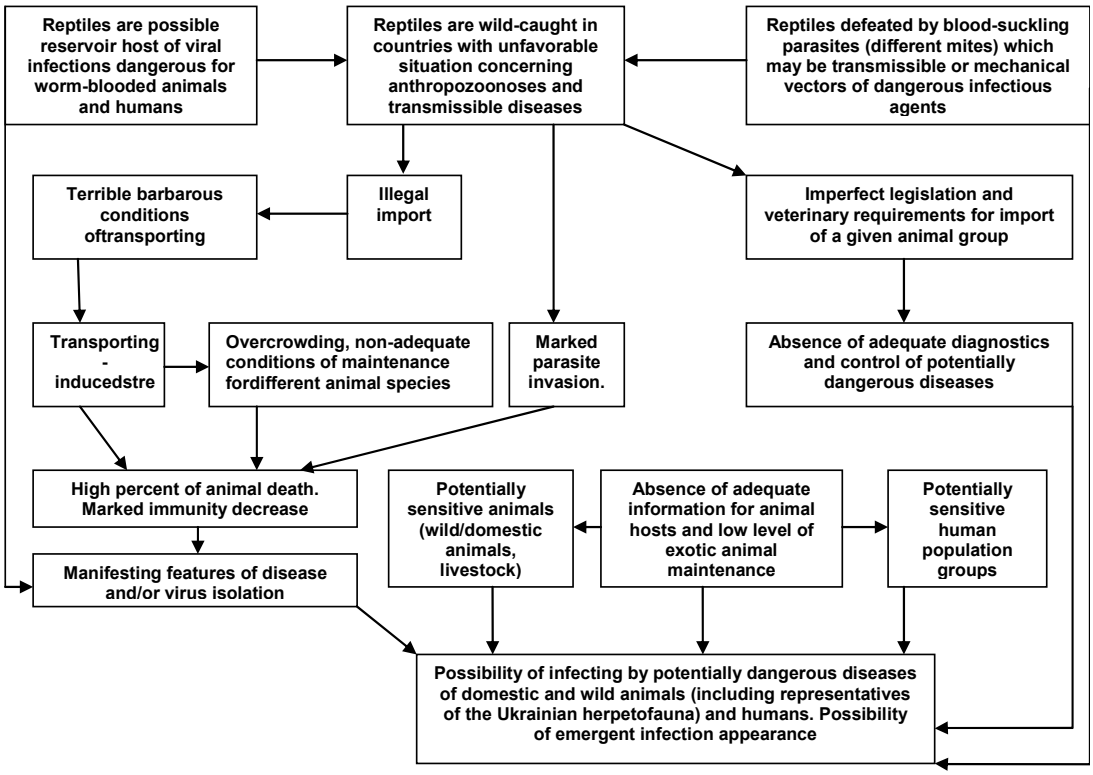
Independent experts consider the annual animal turnover smuggling in Ukraine reaches $200 million [1]. Our country is not only an outlet for such animals, but also their transit, export, and reexport nidus. According to the WWF data, Ukraine is the main transit country concerning wild animal supply from the Western Europe to post-Soviet states and vice versa [1]. The Kyiv Eco-Cultural Center[1] considers more than 10 million of reptiles together with other animals have been illegally imported to Ukraine during last years [1]. According to official statistics, the quantity of arrested illegal imported exotic animal batches in post-Soviet countries become almost twice higher comparing to batches arrested several years ago [16]. The main suppliers of reptiles, amphibians and other exotic animals for Russian and Ukrainian "black market" are countries of South-Eastern Asia, South America, and Africa, most of them being unfavorable from the point of view of dangerous viral infections [16]. Among such infections there are also ones whose vectors and/or intermediate hosts may be also cold-blooded animals (table 2).

***Table* 2. Anthropozoonoses which may be common for cold-blooded, warm-blooded animals, and humans**

| Disease and its agent | Hosts and vectors | Disease manifestations in domestic animals | Source of isolates (of reptile viruses) | Disease manifestations in humans |
|---|---|---|---|---|
| Western equine encephalomyelitis (WEE), a virus of *Togaviridae* family | Birds, squirrels, snakes, horses.<br>Vectors: mosquitoes | Without manifestations in all animals except horses, their lethality being about 30% | Blood samples of tortoises and crocodiles | Encephalomyelitis |
| Eastern equine encephalomyelitis (EEE), a virus of *Togaviridae* family | Birds, some lizards, and crocodiles<br>Vectors: mosquitoes | Without manifestations in all animals except horses, their lethality being about 95% | Antibodies are detected in tegu lizards; some wild alligatos of Mississippi are also proved to be seropositive | |
| West Nile fever (VNF), a virus of *Flaviviridae*family | Toads, snakes, crocodiles, monitors, tortoises.<br>Vectors: mosquitoes | Neurologic symptoms<br>Crocodiles: neurologic symptoms, high lethality | Virus is highly pathogenic for crocodiles; high lethality is seen for young animals. The agent was found in 1hese animals of all continents. | Fever, neurologic symptoms |
| Japanese encephalomyelitis, JEM, a virus of *Flaviviridae* family | Lizards, snakes, birds, horses, pigs.<br>Vectors: mosquitoes | Severe encephalitis<br>No symptoms in reptiles | Field isolates from snakes; development of viremia following an infected insect has been eaten | Encephalitis |

Besides, new emergent infections appeared during last years are a serious threat not only for human-organized, but also for natural reptile and amphibian populations, so we must pay more attention to researches concerning the viruses mentioned above as probable etiologic agents causing these animals infective diseases [3]. Captive animals caught in wilderness find themselves in contact with new pathogens. Stresses due to capture as well as to transporting and non-adequate life conditions cause significant immune suppression, the animals becoming more susceptible to infections. In addition, animals from different continents are kept very oft together, such conditions being exceptionally favorable for infective agents spreading and their inculcation into new host organisms. Without any doubt, the pathogen evolution takes place, these agents becoming able to infect new hosts in which they have not been earlier detected [2,4,10]. Taking into consideration a low level of animal maintenance by their hosts (the worst situation is seen with exotic species) there is a real threat of new pathogen penetration into the environment. Combination of these factors may lead to pathogen spreading among sensible animals and humans as well as to catastrophic results for local herpetofauna if infected individuals penetrate to local natural animal populations (Scheme 1).

**Scheme 1. Possibleways of inter-species transmission of dangerous pathogens and factors favorable for such processes**




The infections being the most dangerous for poikilotherm animals are amphibian chytridiomycosis [34] and ranavirosis (it is now included into the OIE list of dangerous diseases [17]) as well as the spreading of the West Nile virus in Mississippi alligator populations in the USA [18]. There are also reports about diseases of other crocodiles species from different continents [3,18,25,30]; insect diseases caused by agents belonging to the Iridoviridae family are found also to infect reptiles [21,26,27]; there are reports on land and water tortoises infecting by the amphibian ranovirus [20,29]. The infections being the most widespread and causative mass mortality of reptiles in collection include: herpesvirus caused infection of tortoises, paramyxovirosis affecting snakes, and inclusion body disease of boas and pythons. About 12 viral agents reproducing in humans and other mammalian species have been successfully inoculated to reptiles during experimental studies [3, 10].

According to Ukrainian veterinary norms, only bacteriological control (to detect the presence of Salmonella) and quarantine during 30 days are required. However, such preventive measures are not sufficient for detection of many dangerous infections widely spread among wild cold-blooded animals. That is why there is an urgent necessity of elaboration of veterinary approaches intended for early diagnostics and including quarantine measures, screening inspection of recently imported or caught animals as well as organization of effective treatment for a given animal group. The problems discussed above become more and more burning not only for persons being reptile hosts and maintaining them as zooculture objects, but especially for those who take part in the realization of programs intended to conserve and re-introduce exotic and rare species as well as in trade regulation, reptile transporting, and elaboration of veterinary norms for this animal group.

We have begun investigations aiming to obtain primary cold-blooded animal cell cultures in prder to elaborate virus-sensitive cell systems necessary for reptile virus isolation. As cell donors African chameleon *(Chamaeleo calyptratus)* and sand lizard *{Lacerta agilis)* were used. Cell suspensions were prepared by cold trypsinization. Now we aim to determine the most favorable temperatures for cell cultivation (29 °C, 37°C) and optimal culture media compositions (based on DMEM and RPMI 1620 media). Chameleon cell cultures (prepared from kidney tissues) were kept 4 days at 28°C before the beginning of cell proliferation. The medium was then substituted after each 3 days of cultivation, different embryonic calf serum concentrations being added (from 10 % up to 20 %). In 10 days a non-confluent cell monolayer was seen, the cells being similar to lengthened spindle-shaped ones (we have not yet found

cells of such morphology in the available scientific literature) [8]. The cells which had developed no complete monolayer survived during 29 days, their death causes being unknown. A primary cell culture prepared using *Lazerta agilis* kidneys and testicles survived at 29°C during 16 days.

**Conclusions.** The analysis of available published data proves the spreading of dangerous pathogens among reptiles, these pathogens being able to cause viral diseases of warm-blooded animals and humans. It is extremely necessary to pay attention to reptilian viruses investigation among Ukrainian reptilian populations to maintain and increase the biosafety in our country.

1. Березовская Ю.Зверские доходы// Контракты. – 2005. – №20. – С.17-18. 2. Васильев Д., Швед В. Вирусные болезни рептилий// Научные исследования в зоологических парках. – 2007. – №22. – С. 182-215. 3. Килочицкий П., Килочицкая Н., Шеремет В. Динамика популяций кровососущих комаров на территории Киева// I Всероссийское Совещание по проблемам изучения кровососущих насекомых: Матер. науч. конфер., С.-Петербург, 2006. – С. 34-38 http://www.zin.ru/conferences/blsuck1/Tezisy_htm/Kilochitzki.htm. 4. Ariel E.Viruses in reptiles//Veterinary Research. – 2011. 5. Clark F. Growth and Attenuation of Rabies Virus in Cell Cultures of Reptilian Origin// Experimental Biology Medicine. – 1972. – vol.139. – pp.14-18. 6. Cupp E., Zhang D., Yue X., Cupp M., at al. Identification of reptilian and amphibian blood meals from mosquitoes in an Eastern Equine Encephalomyelitis virus focus in central Alabama//J.Trop. Med. Hyg. – 2004. – 71(3). – pp.272-276. 7. Flaviviridae./Thiel H., Collett M., Gould E., at al.// Taxonomy, Eighth Report of the International Committee onTaxonomy of Viruses;Virus Elsevier Academic Press: Amsterdam, The Netherlands., – 2005.- pp. 981–998. 8. Freshney J. Culture of animal cells:a manual of basic technique. – 2005. 9. Infection with ranaviru s/http://www.oie.int/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/Ranavirus_card_final.pdfhttp://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2012/. 10. Jacobson E. Infection diseases and pathology of reptiles. – 2007. 11. Karstad L.Reptiles as possible reservoir hosts for Eastern encephalitis virus//Trans 26th North Am Wildlife Conf. – 1961. – pp.186-202. 12. Komar, N. West Nile virus: Epidemiology and ecology in North America. Adv. Virus Res. 2003,61, 185–234. 13. Marschang R. Viruses Infecting Reptiles [Electronic resource] // Viruses. – 2011 November; 3(11): 2087–2126. Mode of access: WWW. URL: ncbi.nlm.nih.gov http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3230843/ – Title from the screen. 14. Nevarez J., Mitchell M., Kim D., at al. West Nile virus in alligator, Alligator mississippiensis, ranches from Louisiana// H. Herp. Med. Surg.- 2005. – vol.15. – pp. 4-9. 15. Oya A., Doi R.., Shirasaka A., at al. Studies on Japanese encephalitis virusinfection of reptiles. I. Experimental infection of snakes and lizards// Jpn. J. Exp. Med. 1983, 53,117–123. 16. The Reptile and Amphibian Communities in the United States January 2001 USD A: APHIS :VS Centers for Epidemiology and Animal Health.

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## FIRST DETECTION OF HOSTA VIRUS X IN UKRAINE

***В статье представлены результаты мониторинга Х вируса хосты (HVX) в Национальном ботаническом саду Украины им. Н.Н. Гришка. На растениях хосты наблюдали симптомы мозаики и деформации листьев. Присутствие HVX-инфекции в растениях хосты сорта 'Sum of and substance' было подтверждено результатами ELISA, TEM и RT-PCR. HVX был изолирован из больных растений хосты, концентрация очищенного препарата HVX составляла приблизительно 3 мг/мл.***

***The results of Hosta virus X (HVX) monitoring in M.M. Hryshko National botanical garden of Ukraine (NAS of Ukraine) are presented. Leaf mosaic and deformation symptoms were observed on hosta plants. The presence of HVX in hosta cultivar 'Sum of and substance' was confirmed by ELISA, TEM and RT-PCR. HVX was isolated from diseased hosta. Purification of HVX from hosta leaves yielded about 3 mg/ml.***

Hostas or (funkias) are very popular in Ukraine and worldwide, hardy herbaceous perennials grown primarily for their beautiful foliage. Hosta plants generally reache full maturity in 4-8 years, and their size depends on the cultivar [1]. Hosta foliage can be blue, gold (yellow), or green and are often variegated. They are easy to grow and shade-tolerant plants. The plants are low maintenance and are widely available in nurseries and garden centers. In Ukraine hosta settled in private collections, but the biggest collection has been mounted in the National botanical garden by M.M. Hryshko. Certain hosta cultivars are susceptible to a number of viruses, including Hosta Virus X (HVX). Despite its relatively recent discovery, HVX had a significant impact on the hosta industry resulting from the infection and subsequent destruction of many HVX-infected hostas [2]. Symptoms of this virus include mosaic, chlorosis, and necrosis on leaves; severely affected leaves may wither and die. HVX can be transmitted during vegetative propagation of plants and mechanically. Diagnostic laboratories and nursery surveys of symptomatic plantshave confirmed a number of HVX-infected hosta cultivars worldwide [3]. New HVX-infected hosta cultivars continue tobe detected. Investigations of distribution of HVX in Ukraine have not been conducted. The aim of this work was monitoring of HVX in M.M. Hryshko National botanical garden of Ukraine.

**Materials and methods.** During autumn 2011, the samples of hostas exhibiting virus-like symptoms were collected from M. M. Gryshko National Botanical Garden (NAS of Ukraine). Several samples were collected from symptomless plants. A total 21 samples from 4 hosta cultivars ('Sum and substance', 'Striptease', 'Lady Guinevere', 'Old Faithful') were analyzed for the presence of the virus.

Taking into account serological relation between HVX and PVX (potato virus X) indirect ELISA tests of hosta samples were carried out using antiserum to PVX.

For EM examination, partially purified plant sap was mounted on formvar grids and negatively stained with 2% uranyl acetate. The preparations were viewed under the electron microscope at an instrumental magnification of 20,000.

The method used or virus purification was adapted from those of Goodman (1975) for PVX. The virus was purified by clarification in chloroform, precipitations using polyethylene glycol 6000, and differential centrifugation.

Total RNA was extracted from plant material and subjected to RT-PCR using primers a6448 and s5722 corresponding to nucleotide sequences 6448-6428 and 5722-5742, respectively of HVX from Korea (HVX-Kr) (GenBank Accession No. AJ620114)[1,3].

**Results and discussion.** The main objective of the present investigation was diagnostic of hosta plantings for infection with HVX in Ukraine. For this purpose four varieties of hosta were tested. Visual monitoring of hosta plantings revealed the presence of plants with symptoms of viral disease. On hosta plants of cultivar 'Sum of and substance' we observed dark, green streaking and puckering along the leaf veins (Fig. 1.). The plants of cultivars 'Striptease' and 'Lady Guinevere' with symptoms of chlorosis and leaf desiccation were collected for farther analysis (Fig. 2,3). Hosta 'Old Faithful' displayed puckering and distortion on leaves (Fig. 4.). According to the literature data the symptoms of HXV-infection can vary depending on cultivar, plant age and growth condition. The viral symptoms also can be confused with symptoms cased by other ethiological agents. To reveal viral etiology of diseases more specific serological methods should be used.

**Fig.1. Virus-like symptoms on hosta of cultivar 'Sum of and substance'**

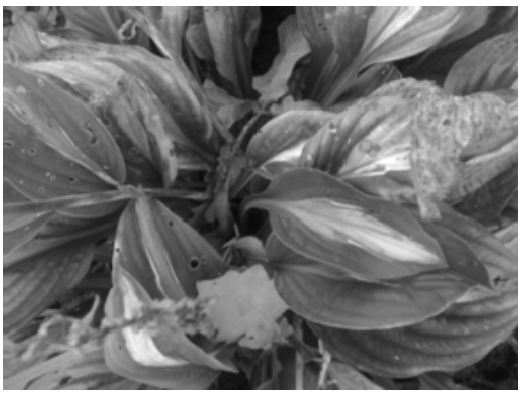

**Fig.2. Chlorosis and leaf desiccation on hosta of cultivar 'Striptease'**

**Fig. 3. Color blotches and leaf desiccation of hosta 'Lady Guinevere'**

**Fig. 4. Puckering and distortion on leaves of hosta 'Old Faithful'**

HVX belongs to the genus potexvirus and is serologicaly related to PVX so it was a good chance that newly isolated HVX would react with antiserum raised against PVX. Given this we used anti-PVX antiserum for primary identification of HVX in collected samples. According to ELISA results HVX was detected in pants of 'Sum of and substance' cultivar (Fig. 5), however absorbance value in this case was low. The samples of other hosta cultivars gave negative results is ELISA.

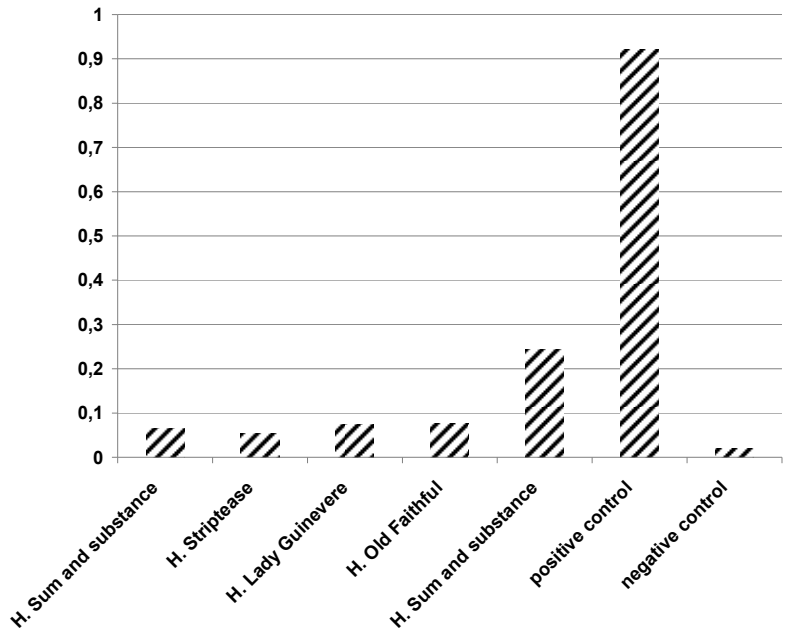


**Fig.5. ELISA results for HVX detection in the samples of hosta**

Transmission electron microscopy revealed the presence of filamentous viral particles in partially cleared plant sap obtained from hosta of cultivar 'Sum of and substance' (fig.6). The modal length and diameter of such particles were 470-580 and 13 nm, respectively. According to virion morphology and size observed viral particle correspond to HVX.

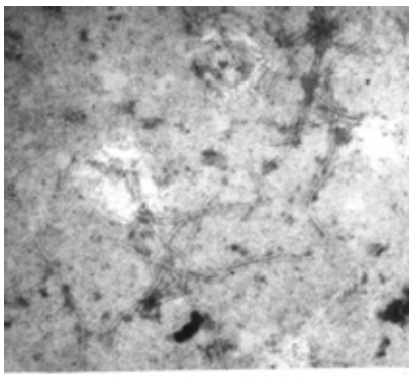

**Fig.6. Electron micrographs of virus isolated from hosta (instrumental magnification 20,000)**

The virus was purified from systematically infected of hosta plants (cultivar 'Sum of and substance' ). An yield of 3,1 mg /ml was thus obtained. The 260/280 ratio of the purified preparation was 1.37. Purified virus will be used for the future research of isolated virus and for obtaining diagnostic antiserum.

Taking into account low absorbance values obtained under ELISA we performed RT-PCR for detection of HXV in collected samples. At raising of RT – PCR used kit SYPER – SKRIPT – 2(Invitrogen, USA), and pair of primers that amplification product by molecular mass 706[9]. We modified methodology of raising for reaction. On the first stage conducted a selection to the virus(but not RNA). After warming up, the standards of virus were used for raising of RT – PCR (without a previous selection to RNA). As a result by the method of electrophoresis in 1,5% agarose gels are educed product of amplification of corresponding molecular mass (fig. 7).

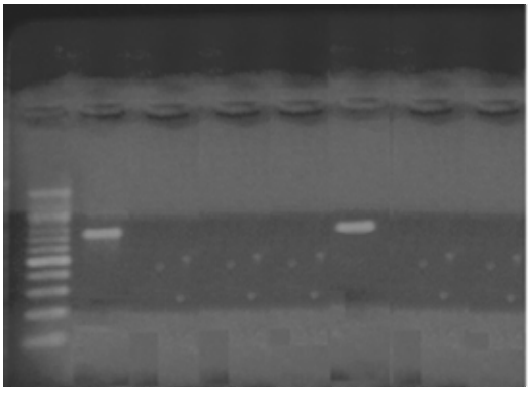


**Fig.7. Agarose gel electrophoresis of RT-PCR products from hosta plants.**
M – markers, 1 -Hosta ' Sum and substance', 2 – hosta 'Striptease', 3 – hosta 'Lady Guinevere', 4 – Hosta 'Old Faithfu', 5 – is positive control, 6 is negative control

The presence of HVX in hosta cultivar' Sum and substance' was confirmed by RT-PCR using a specific primer pair that amplifies the HVX coat protein (fig. 7). The amplified product with corresponding molecular mass (706 bp.) was obtained and will be used for the nucleotide sequence determining of CP-gene of HVX Ukrainian isolate and for phylogenetic analysis. The virus was not detected in samples of another hosta cultivars.

**Conclusions.** Monitoring of hosta virus X in M. M. Gryshko National Botanical Garden revealed the presence of this virus in at least one hosta cultivar. According ELISA and RT-PCR results another three investigated hosta cultivars were free from HVX. However all this cultivars displayed the symptoms of viral infection. Hosta plants can be infected with several viruses including impatiens necrotic spot virus, tomato ringspot virus, tobacco rattle virus. The causative agent of virus-like symptoms in this case should be established using specific methods.

Identified HVX was purified from plant material and will be used for obtaining antiserum with in turn will enable a diagnostic of this virus in Ukrainian collections of hosta. Hosta is a popular perennial ornamental plant in Ukraine. Hence, measures should be taken to reduce HVX spread. Screening of hostas for HVX prior to distribution is the first important step in control of this virus.

1. Ryu KH, Choi SH. Molecular detection and analysis of Sweet potato feathery mottle virus from root and leaf tissues of cultivated sweet potato plants // Plant Pathol. – 2002. – Vol 18. – P 12–17. Currier S, Lockhart BEL. Characterization of a potexvirus infecting Hosta spp // Plant Dis. – 1996. – Vol 80. – P. 1040–1043. 2. Ryu KH, Park MH, Lee JS (2002) Occurrence of mosaic disease of Hosta plants caused by Hosta virus X // Plant Pathol. – Vol 18. – P. 313–316. 3. Ryu KH, Min BE, Choi GS, Choi SH, Kwon SB. Zucchini green mottle mosaic virus is a new tobamovirus; comparison of its coat protein gene with that of kyuri green mottle mosaic virus // Arch Virol. – 2000. – Vol 145. – P. 2325–2333.

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**O. Shevchenko, PhD**

# SUMMARY OF LAB DIAGNOSTICS FOR VIRUS PESTS ENDANGERING GLASSHOUSE FARMING OF VEGETABLES IN UKRAINE: THE OUTCOMES, SOURCES OF INFECTION, AND CONCLUSIONS

***Данная работа обобщает результаты проведенных лабораторией научных экспертиз растительного, семенного и посадочного материалов овощных культур, которые выращиваются в тепличных условиях. Приведены данные относительно инокулюма и рекомендации для предотвращения развития вирусных инфекций.***

***This work summarizes the outcomes of conducted laboratory testings of plant, seed and seedling material for vegetable cultures grown in glasshouse conditions. Here we provide data of the source(s) of infection and recommendations regarding preventive measures against virus diseases.***

**Introduction.** The Department of Virology at Taras Shevchenko' Kyiv National University is a known expert in monitoring and diagnostics of virus diseases of agricultures and in plant virus epidemiology. During the last decade we have been extensively involved in studying virus diversity and spread for both natural and manmade ecosystems for fundamental issues (virus ecology, virus epidemiology and evolution). This is reflected in vast number of publications [1-4] and related joint international projects with leading institutions from Czech Republic, Germany, Bulgaria, Hungary, Turkey, Belarus and the USA.

Glasshouse farms represent so called 'closed ground' systems and having stable temperature, light and humidity conditions are posed to significantly higher risk of development of bacterial, virus and fungi diseases. Artificial conditions of glasshouses not only favor propagation of plant virus vectors – insects and nematodes – but also seriously intensify mechanical transmission of the pathogens due to the elevated density of plants and fungi growing there. Because of indicated reasons, mixed infections of plants or fungi with viruses and other pathogens are rather common for glasshouses and endanger the commercial outcomes of farming. Analysis of plant/seed material and soil, fungi and substrate for viruses will allow reacting adequately, introduce prophylactic measures and minimize losses of commercially valuable production.

During the last 5 years we have faced a growing demand from conscious farmers looking for possible means of improvement of their farming business. Glasshouse farming has become an important branch of agriculture in Ukraine and is growing with a very fast pace. Among the major plant cultures grown in glasshouse conditions are typical vegetables: tomato, cucumber, sweet pepper, eggplant, cabbage and zucchini. It is worth saying that new glasshouses are mostly state-of-the-art both in terms of the structure itself and in terms of technology, and virtually every possible measure for plant cultivation (the light, watering regime and technologies, use of special substrates, nutritional norms, etc.) and defense (use of certified seed material, use of sterile substrate, vector control, etc.) has been put into place.

In spite of such measures, we are regularly contacted by farmers asking to check their plant/seed/substrate material for viruses infecting different cultures. Here we represent major outcomes of such experiments and provide our conclusions.

**Materials and methods.**

*Sample preparation*

Samples of vegetative tissues were homogenized in sterile mortars with autoclaved 0.1M PBS buffer, pH 7.4 (1:2-1:4, w/v), at 4˚C. Then the extracts were subjected to low-speed centrifugation at 5000 rpm for 20 min (4˚C) on RS-6 centrifuge ('TNC DASTAN', USSR) to remove the debris [5]. Obtained clarified extracts were further used for virus detection via ELISA.

In case of seeds, these were soaked in sterile water at room temperature for 24-72 hours and then treated as described above. Soil and substrate samples have been homogenized the same way.

*ELISA*

We have used DAS-ELISA as recommended by EPPO [6]. In this work we have employed commercial antisera for different viruses of Loewe and DSMZ (Germany), Prime Diagnostics (The Netherlands), and INRA (France) following the manufacturers' instructions.

Briefly, 96-well sterile plates ('Labsystems', Finland) were coated with unconjugated virus-specific antibodies and incubated for 4 h at 36˚C to allow to adhere to the wells. Then the samples (clarified sap) were deposited and incubated overnight at 4˚C. Then the conjugated virus-specific antibodies were applied and incubated for 4 h at 36˚C. Each step was followed by triple washing of the plates with 0.2% Tween in 0.1M PBS, pH7.4. The last step was the application of substrate solution (n-nitrophenyl phosphate) for alkaline phosphatase ('Sigma', USA) to visualize positive reaction, and incubation for 60 min at room temperature in the dark. The reaction was stopped with 3M NaOH. Finally, the results were counted at the wavelength of 405 nm (OD405) on ELISA reader ('Dynatech', Germany).

**Results and discussion.** Among the major cultures grown in glasshouses are: *Lycopersicon esculentum*, *Capsicum annuum*, *Cucumis sativus*, *Solanum melongena*, *Brassica oleracea* and *Cucurbita pepo* (zucchini). These can be infected by a vast list of viruses. We have carried out the testing for major and most dangerous viruses only, which are most typical for these plants and found regularly in Europe (and Ukraine) both in glasshouses and open fields. The results are given in Table 1.

***Table* 1. Summarized outcomes of testing of major vegetable cultures grown in glasshouse conditions for virus diseases (2008-2012 yy)**

| Culture | Viruses found | Overall percentage of infected plants | Tentative source of infection |
|---|---|---|---|
| *Lycopersiconesculentum* | CMV, PMMV, TRV, TMV, TRSV, AMV | 5-50% | Mechanical transmission or vector |
| *Capsicumannuum* | CMV, AMV, PMMV, ToMV, TMV, TRSV, TSWV | 5-60% | All possible means of infection |
| *Cucumissativus* | CGMMV, CMV | 10%-100% | Mechanical transmission, seed material (CMV) |
| *Solanum melongena* | CMV, TRV, TSWV, TRSV, AMV, ToMV | 10-90% | All possible means of infection |
| *Brassicaoleracea* | AMV | 5-50% | Mechanical transmission or vector |
| *Cucurbita pepo (zucchini)* | ZYMV, CMV, WMV-2 | 10-100% | Vector or mechanical transmission, seed material (CMV) |

As can be seen from this table, many viruses are found to invade these cultures in glasshouses. As normally only diseased (symptomatic) plants are brought into the lab for subsequent analysis, the infection rate varies from moderate (5-10%) to high or extreme (50-100%).

We should also say that relatively many plant samples (up to 10%) were infected with 2 viruses or more (mixed infection).

Analyzing the sources of infection(s) we came to surprising conclusion. In most cases the seeds of respective cultures were virus-free, which is rather logical as the seeds are mostly imported being certified as virus-free. There are several exceptions and *Cucumissativus* seeds are the most prominent example here. Several batches of seeds of this plant obtained from different sources (i.e., farmers) contained CMV, and they were all imported from Russia.

The most common source of infection, again surprisingly, was mechanical transmission of the virus in the glasshouse. In such a way, something (tools? equipment?) or somebody (workers) brought the virus in the glasshouse where the initial infection then happened. Further on, initially infected plant has become a source of virus for its neighbours.

Vectors (flying insects, predominantly aphids) are a rare case as can be relatively easily monitored and controlled.

Analysis showed that soil and substrate are uncommon sources of infection, as they are regularly changed (substrate) or sterilized (soil).

Insofar we may conclude that major problems with viruses we encounter in glasshouse farming are the non-compliance with hygienic and sanitary norms.

**Conclusions.** In this work we have demonstrated that plants cultivated in 'controlled' glasshouse conditions are often infected by different 'uncontrolled' viruses. Surprisingly, even when using certified virus-free seed material and controlling vector populations in the glasshouse (virtually every farmer has confirmed that they have no vectors at all!) we still can see rather high level of virus infections in major vegetable cultures. This is reflected in millions of dollars lost annually in total.

Therefore, the neglect of proven hygienic and sanitary norms is the major cause of such losses. There two points every farmer can do in order to improve this situation, ensuring:

1) proper training of its personnel;

2) regular random-based diagnostics of plant material for most common viruses.

Sporadic testing (that's what our lab does, when we've sent already diseased plants for testing) can only witness moderate of high level of infection. Regular analysis will help eradicate diseased plants when they are not a source of infection yet (i.e., when there are no symptoms yet, and hence there's no reason to eradicate such plant).

Proper knowledge and conducting diagnostics in advance will help avoiding crop losses in future.

In addition, we may add that the cultivation of plants in the open field faces similar problems. However, the typical rate of infection for such plants is much lower. Same recommendations apply here as well – be prepared and act in advance.

Our rich experience, trained scientific personnel and state-of-the-art equipment for plant virus diagnostics allow to propose:

Diagnostics of virus diseases:

- Cereals (rice, wheat, barley, rye, oat, maize)
- Oil and industrial cultures (sunflower, cotton, sugar beet, sugar cane, rape, tobacco)
- Vegetableand cucurbitaceous cultures (potato, tomato, cucumber, pepper, eggplant, zucchini, marrow, melon, cucurbit)
- Legumes (soybean, bean, pea)
- Fruitcultures (apple, prune, cherry, sweet-cherry, реасняблуня, слива, вишня, черешня, персик)
- Decorative and aromatic cultures (orchid, rose, carnation, lavender).

Virus diagnostics may be carried out in plant material, soil, etc. Knowledge about which virus infections prevail in Your crops will allow You conducting: i) better and more reliable planning of land use and crop rotation, ii) optimal selection of virus-resistant or tolerant plant variety, iii) define most proper time for culture planting and harvesting. Moreover, virus diseases are prerequisites for further developments of fungal and bacterial pathologies, and hence their timely diagnostics would enable You to utilized plant protection means more efficiently.

Testing seed material on virus infections

Many virus diseases are efficiently transmitted by plant seeds. This is of special importance for wheat, rice, rye, barley, sugar beet, sunflower and legumes. Presence of virus in the seed pose a serious problem as every plant grown from this seed stock will be infected. Furthermore, seed control for the absence of virus pathogens is an acting international standard of sanitary epidemiological control of plants. Diagnostics of seed material in our lab will save You time and valuable relationships with Your partners.

Obtaining of virus-free planting material

In our lab, we adopted techniques for microclonal propagation of plants. The technology allows obtaining virus-free plant material even from virus-infected plants. Such approach is the only reliable mean to preserve unique decorative plants including roses, carnations, etc. The technique enables commercial growing rare or disappearing plant species independently from the season of the year. Microclonal plant propagation also allows to re-establish collections of planting material for genetic selection of agricultures.

Training and professional development in:

- Visual monitoring of crops of agrarian and decorative cultures for virus diseases
- Evaluation of pathogen spread on a given territory

- Methods for complex diagnostics of plant virus infections (theoretical and practical training)
- Analysis of plant material for presence of virus pathogens utilizing serological techniques (enzyme-linked immunosorbent assay, its modifications)
- Analysis of plant material for presence of virus pathogens utilizing molecular techniques (polymerase chain reaction, its modifications)

- Consulting servicesin diagnostics and monitoring of plant virus diseases
- Raising of specific antiserums to common plant viruses (for Your use inself-dependent serological diagnostics of virus disease via enzyme-linked immunosorbent assay)
- Design of molecular probes to any plant virus (for Your use inself-dependent serological diagnostics of virus disease via polymerase chain reaction).

Working with us, You may rely on our individual approach to Your needs, timely and accurate accomplishment of the work, and mutual benefit as reflected by our friendly and long-lasting relations with major plant growers in Ukraine.

1. Dmitriev A., Shevchenko O., Polischuk V., and Guscha N. 2009.Effects of low dose chronic radiation and heavy metals on plants and their fungal and virus infection. Data Science Journal, 8(24): 49–57. 2. Polischuk V.P. 2005. Effects of radioactive and chemical pollution on plant virus frequency / V.P. Polischuk, T.P. Shevchenko, I.G.Budzanivska [et al]. NATO Security through Science Series, Environmental Security: Vol.2., Equidosimetry (F. Brechignac and G. Desmet, eds.). P.87-92. 3. Polischuk V.P., Budzanivska I.G., Shevchenko T.P. Abiotic environmental factors: effects on epidemiology of plant virus infections Cooper J.I. et al. (eds.).2006. Virus Diseases and Crop Biosecurity. NATO Security through Science Series, Springer. P.121-132. 4. Dmitriev A.P., Shevchenko O.V., Polischuk V.P., Guscha N.I. Chapter XVII: Effects of low dose chronic radiation and heavy metals on plant-pathogen interactions, pp.229-243. In: Burlakova E.B., Naydich V.I. (eds.) The lessons of Chernobyl: 25 years later. – Nova Science Publishers, 2012. 5. Hill S.A. Methods in plant virology. Oxford: Alden Press, 1984. 167 p. 6. http://www.eppo.int/.

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**V. Torop, leading engineer, G. Yanishevs'ka, leading engineer, O. Bisyarina, student, O. Kondratyuk, PhD**

## ASSESSMENT OF THE REACTION OF WHEAT TO THE STREAK MOZAIK VIRUSES INOCULATION ACCORDING TO MALONDIALDEHYDE CONTENT

***Изменения перекисного окисления липидов в растениях озимой пшеницы сорта "Память" оценивали на содержание малонового диальдегида в 1 и 14 день после инфицирования вирусом полосатой мозаики пшеницы. Были обнаружены специфические черты реакции в корнях и листьях при инокулировании растений вирусом. Соотношение между содержанием малонового диальдегида в листьях и корнях могут быть использованы для ранней непрямой диагностики инфекции.***

***Changes in lipid peroxidation in plants of winter wheat variety "Pamyat'" were evaluated for content of malon dialdehyde in 1 and 14 days after infection with the streak mosaic virus. The special features of the reaction in the roots and leaves to the inoculation with the virus were discovered. The relationship between malon dialdehyde content in leaves and roots can be used for early indirect diagnosis of the infection.***

Patosystems with wheat streak mosaic virus (WSMV) became often the object of successful research involving both fundamental and applied areas of virology. The application of methods assessing the physiological state of plants is important in a comprehensive approach for determining sensitivity of host plants, the indirect, rapid diagnosis of viral infection [5]. Malondialdehyde (MDA) that is one of the end products of lipid peroxidation (LPO) is also one of the universal criteria for early diagnosis of stress [2–4]. Therefore, the MDA content is a convenient measure of plant response to the initial action as abiotic and biotic factors.

**The aim** of our work is evaluating the physiological state of plants of wheat according to the contents of MDA under the conditions of artificial inoculation WSMV.

**Materials and methods.** The seeds of winter wheat variety "Pamyat'" were treated potassium permanganate solution. The plants were grown on moistened paper in Petri dish, covered glass microbiological caps with Knopp solution added. Two-weeks plants were infected WSMV. The MDA content was determined based on the reaction of 2-tiobarbiturova acid [1, 6]separately in the leaves and roots after 1 day and 14 days after inoculation. The difference between variants of the experiment is accurate at $p<0,05$.

**Results and discussion.**The nature of infection and its symptoms in model systems is somewhat different from those observed in agrocenoses under the conditions of natural infection. This is largely due to the influence of buffer solution and carbide which are used for artificial inoculation of plants. In our experiment plants of winter wheat variety "Memory" even on the 14th day after the infection did not show the external signs of disease, although after artificial inoculation characteristic symptoms are usually recorded earlier than in the field. However, by changing the intensity of LPO the signs of stress caused by WSMV could be found within 18 hours after inoculation (fig. 1).

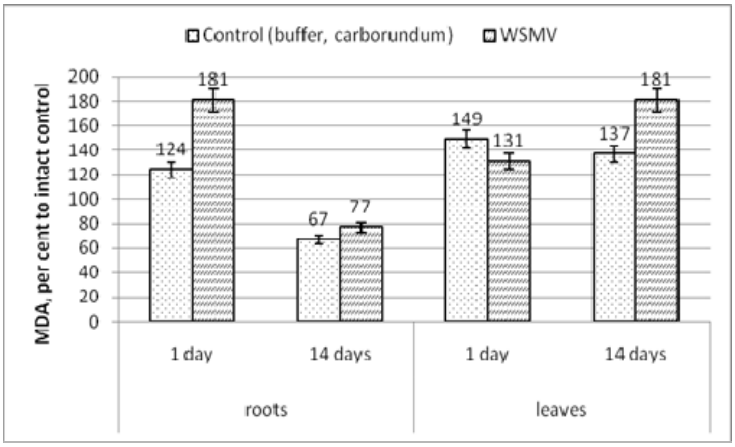


**Fig.1. Lipid peroxidation (MDA content) in winter wheat plants variety "Pamyat'" as correlation (% to intact control) between MDA contents on 1 day and 14 days after inoculation by WSMV ($p<0,05$)**

In the roots of infected plants MDA content was increased by 57%, in the leaves – on the contrary, decreased by 18% compared with control (buffer solution, carborundum). On the 14th day MDA level increased by 44% in the leaves of infected plants relative to experimental controls and in the roots it was higher only by 10%. In intact leaves the content of MDA in the beginning and end of the experiment was 0,430-0,436, in the roots – 0,134-0,140 mkM / g of crude material. The content of MDA in the leaves of control (intact) plants was usually three times higher than in roots (fig. 2).

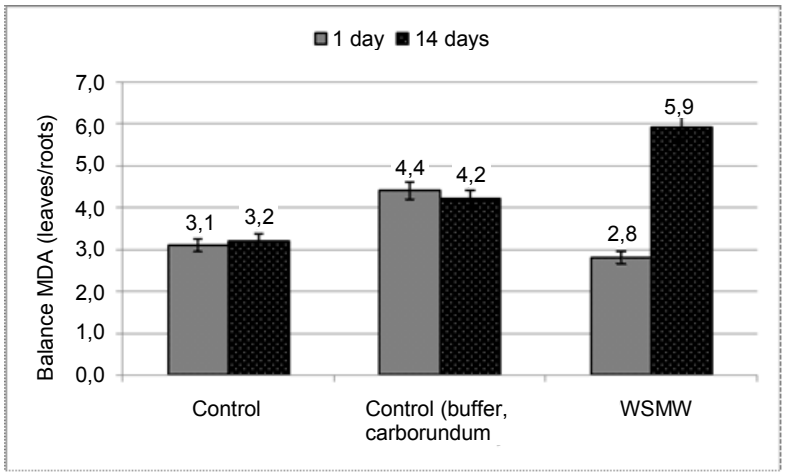


**Fig.2. Lipid peroxidation (MDA content) in winter wheat plants variety "Pamyat'" as balance MDA content (mkM/g fresh weight) (leaves/roots) on 1 day and 14 days after inoculation by WSMV (p<0,05)**

14 days after infection, MDA in the leaves exceeded its value in the roots six times. In the control (buffer, carborundum) plants one day after exposure to abiotic factors – four times. After 14 days the leaves/ roots LPO balance came to normal. A wide range of changes in MDA content indicates the reaction of wheat plants to a compatible action of abiotic and biotic factors in inoculation. The balance between lipid peroxidation and antioxidant defense may characterize indirectly the infectious properties of the pathogen and the tolerance of the sort [2–4].

**Conclusion**Thus, the evaluation MDA content at the same time in both leaves and roots of infected plants allowed within 1 day after inoculation to detect early response of plants to WSMV.

The assessment of leaves/ roots LPO balance allows to distinguish actual effect from WSMV influence from the effect of abiotic factors, which are violations caused to plants by artificial inoculation. The range of the detected changes may indirectly characterize the sensitivity of plants to oxide stress caused by infection, displays the features of virus transport and dynamics of its development in time.

1. Velikova V., Yordanov I., Edreva A. Oxidative stress and some antioxidant systems in acid rain treated bean plants. Protective role of exogenous polyamines // Plant Science. – 2000. –V. 151. – P. 59-66. 2. Гарайда О., Кобилецька М., Терек О. Активність супероксиддисмутази та вміст перекису водню у проростках за дії саліцилової кислоти й фітопатогенну // Біологічні студії. – 2010. – Т.4, №3. – С.63–70. 3. Дмитрієв О.П., Кравчук Ж.М. Активні форми кисню та імунітет рослин // Цитолоія і генетика, 2005. – №4. – С. 64–74. 4. Казначеєва М.С., Цебржинський О.І., Вміст малонового діальдегіду в сортах рослин, різних за рівнем стійкості до хвороб // Вісник ОНУ. – Т. 16. Вип. 6.–2011. – С. 12–17. 5. Міщенко Л.Т., Решетнік Г.В., Тороп В.В. Комплексна діагностика симптомів багряних листків озимої пшениці. – Вісник аграрної науки, 2010. – С. 27–30. 6. Стальная И.Д., Гаришвили Т.Г. Метод определения малонового диальдегида с помощью тиобарбитуровой кислоты // Современные методы в биохимии / под ред. В.Н. Ореховича. – М.: Медицина, 1977. – С.66–68.

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## NOVEL VIRAL PATHOGENS OF VEGETABLE CROPS IN UKRAINE AND BELARUS

***Методами иммуноферментного анализа и электронной микроскопии были идентифицированы 8 видов вирусов среди овощных культур в Украине. Три вируса: Cucurbit aphid-borne yellows virus (CABYV), Zucchini yellow mosaic virus (ZYMV) и Impatiens necrotic spot virus (INSV), были детектированы на Украине впервые за последние 10 лет мониторинга. Также впервые были детктированы PMMV and TuMV на сладком перце и томатах. В Белоруссии мы идентифицировали 3 вида вирусов на огурцах и 7 видов вирусов на томатах.***

***Serological and electronicmicroscopy data identified 8 viruses infecting vegetable crops in Ukraine. Three viruses: Cucurbit aphid-borne yellows virus (CABYV), Zucchini yellow mosaic virus (ZYMV) and Impatiens necrotic spot virus (INSV) have been detected in Ukraine for the first time during the 10-year monitoring. We also detected PMMV and TuMV on sweet pepper and tomato for the first time in Ukraine. In Belarus, we have identified 3 virus species on cucumbers and 7 virus species on tomato.***

**Introduction.** Novel data of recent years suggest the expansion of the areal of harmful viral diseases, spread of complex and latent infections, and appearance of new virus forms with altered pathogenicity [1]. Here we analyzed spread of viruses common for vegetable crops belonging to *Cucurbitaceae* and *Solanaceae* families and grown in an open ground and greenhouse conditions in Ukraine and Belarus. Cultivated plants with virus-like symptoms of the disease were sampled including cucumbers, cucurbits, zucchini, melons, watermelons, tomatoes, sweet pepper and egg-plants. Analyzing samples of *Cucurbitaceae* plants we noted the symptoms of dark green mosaic, mottling and vein clearing of leaves, fruit malformation and wartiness. The *Solanaceae* plants demonstrated the following symptoms: yellowing of leaves, yellow and dark mottling, fruits discoloration and malformation.

The work has been focused on analyzing the state of the spread of virus diseases of vegetable crops in

Ukraine and Belarus and revealing tentative novel pathogens of virus nature.

**Materials and methods.** Plants of cucumbers, tomatoes, sweet pepper, egg-plants, etc. with pronounced symptoms of virus etiology were the major objects of this work. The plants have been sampled from various greenhouse farms in Ukraine and Belarus. Open field studies were mainly focused on cucurbit crops and their viruses.

For ELISA, the plant samples have been homogenized with 0.1M PBS + 0.001MEDTA at 1:2 (m/v) with following sedimentation at 4,000 rpm for 20 min at 4°Cusing the PC-6 centrifuge (USSR)[2]. The homogenate was further used for ELISA.

The ELISA has been conducted in DAS modification [3] using the commercial antisera (Loewe (Germany), Prime Diagnostics (The Netherlands), Agdia (USA)) against 15 viruses known to infect vegetable crops in Europe. Manufacturer's instructions were applied.

The samples shown virus-positive in ELISA were then analyzed via transmission electronic microscopy. Partially purified virus preparations were applied onto copper grids coated with 0.2% Formvar (Serva, Germany). Further, the samples have been contrasted with 2% uranyl acetate (Serva, Germany) for 2 mins [4]. Virus preparations were viewed at the instrumental magnification of 20000-60000 on JEOL Jem 1230 microscope (Japan).

**Results and discussion.** According to the results of ELISA, eight viruses has been detected in the symptomatic samples of cucurbit and solanaceous plants grown both in open field and greenhouse conditions in Ukraine. On plants from *Cucurbitaceae* family which were cultivated in the open field we have detected following pathogens: *Zucchini Yellow MosaicVirus* (ZYMV), *Cucumber Mosaic Virus* (CMV), *Watermelon Mosaic Virus* 2 (WMV-2), and *Cucurbit Aphid-borne Yellows Virus* (CABYV). In the greenhouses the following viruses have been detected: *Cucumber Green Mottle Mosaic Virus* (CGMMV) and *Impatiens Necrotic Spot Virus* (INSV). In case of plants belonging to *Solanaceae* family, we have demonstrated the occurrence of *Pepper Mild Mottle Virus* (PMMV) and *Turnip Mosaic Virus* (TuMV).

In addition, we have conducted the electronic microscopy studies of selected samples (Fig.1).

**Figure 1. Electronicmicroscopy of PMMV (instrumental magnification 20000)**

During the 10-years monitoring of viruses infecting vegetables in Ukraine, ZYMV and CABYV have been detected on cucurbit plants grown in the field conditions for the first time. Both pathogens have been identified in the field in Kyiv, Poltava and Odessa regions – major agricultures areas for cultivating cucurbit cultures. ZYMV have been regularly found on squash and zucchini plants. Virus symptoms involved burrs on fruits, dark green stripes along the leaf veins and yellowing of leaf blades.

CABYV infection have been mostly typical for cucumber, squash and melon plants with mosaics and yellowing of leaf blades. Virus infection induced by INSV have been firstly described on cucumbers in greenhouse farm in Donetsk region. Symptoms included dark green mosaics along the leaves with leaves' discoloration.

PMMV and TuMV have been detected for the first time in Ukraine on sweet pepper and tomatoes – typical representatives of *Solanaceae* family. PMMV infected plants in the field conditions invoking following symptoms: yellow-green leaf mosaics, local superficial necroses on fruits, and partially anthicyanic color of fruits. On the contrary to PMMV, TuMV has been detected in greenhouse conditions only.

We should indicate that identified pathogens were detected on *Cucurbitaceae* plants both in mono- and in mixed infections, normally represented by a couple of viruses. For instance: WMV 2/CABYV, WMV 2/CMV, CMV/CABYV, ZYMV/CABYVandWMV 2/ZYMV. According to obtained outcomes, WMV 2, CABYV and CGMMV are the most widespread viruses infecting vegetables in explored parts of Ukraine. The detection of five new (for Ukraine) viruses (ZYMV, CABYV, INSV, TuMV and PMMV) may be attributed to the use of imported seed material and the spread of the insect vectors, but primarily underlines the absence of valid data on virus spread and necessitates the need for careful monitoring of viral pathogens.

As a result of the research carried out in 2009-2012 in Belarus, on the basis of ELISA on tomato plants cultivated in conditions of protected ground we have identified 7 virus species: *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Tomato mosaic virus* (ToMV), *Tomato aspermy virus* (TAV), *Pepino mosaic virus* (PepMV), *Tobacco Rattle Virus* (TRV) and *Tobacco ringspot virus* (TRSV) for the first time in Belarus; on cucumber plant we have registered 3 virus species: TMV, CMV, and *Cucumber green mottle mosaic virus* (CGMMV). Virus-infected tomato plants demonstrated change of leaves' coloration and leaves' deformation (thread-like and fern-like). First symptoms on cucumbers were revealed during seedling period in the form of vein lightening, and later – slight mosaics. During plant fruit bearing, the fruits also got mosaic coloring, were bent and became warty. At the end of a vegetative period masking of disease symptoms was observed, but symptoms of mosaics, mottling and deformation were shown on apical and young leaves of growing shoots. Cultivation of a wide spectrum of tomato and cucumber hybrids, infected by virus diseases in a various degree, can lead to different strains of virus pathogens selection, more adapted for concrete plant genotypes and to loss of their resistance.

**Conclusions.** Here we inform on the appearance and wide spread of five novel viruses endangering vegetable

crops in Ukraine. The detection of five emerging virus diseases – ZYMV, CABYV, INSV, TuMVand PMMV – can be partially attributed to the activation and spread of their respective insect vectors. From the other side, elevated air temperatures and humidity, absence of natural counterparts and other factors favor massive multiplication of the pests in greenhouse conditions. Vector-assisted transmission is highly efficient for these viruses and hence major efforts must be exerted on the controlling of insect vectors via various insecticides. Issues underlining the necessity of improvement of phytosanitary measures must also be raised.

1. Virus taxonomy. Eight report of the International Committee on Taxonomy of Viruses [M. Fauquet, M.A. Mayo, J. Maniloff et al.]. – London: Academic Press, 2006. – 1259 p. 2. JeanneDijkstra; CeesP. deJagerPracticalplantvirology: protocolsandexercises / Berlin; Heidelberg; NewYork: Springer, – 1998, – 459 p. 3. Hill S.A. Methods in Plant Virology, Alden Press, Oxford, Great Britain, – 1984, – P.164. 4. Crowther J.R. ELISA. Theory and practice, Hamana Press, N.Y. – 1995. – P.223.

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# EFFECT OF LOW TEMPERATURE AND EXOGENOUS HYDROGEN PEROXIDE ON THE REACTION OF WHEAT, INFECTED VIRUS

***В данной статье было исследовано влияние экзогенного пероксида водорода, низких положительных и отрицательных температур на растения озимой пшеницы. Иммуномодулирующие воздействие абиотических факторов на растение-хозяина при вирусной инфекции детектировали на основе оценки перекисного окисления липидов, по содержанию малонового диальдегида, реакции роста и ELISA. Обнаруженные соотношение может быть полезно для прогнозирования эпифитотии, вызванные изменением погодных условий, и для разработки экологически безопасных систем защиты сельскохозяйственных культур.***

***The effect of exogenous hydrogen peroxide, low positive and negative temperature on the plants of winter wheat, inoculated with the streak mosaic virus, were investigated. Immune-modulation effect of abiotic factors on plant-host of viral infection was detected based on the assessment of lipid peroxidation according to malon dialdehyde content, growth responses, ELISA. The discovered ratio may be useful for predicting epiphytotics caused by weather changing and the development of environmentally safe protection system in crops.***

Wheat streak mosaic virus (WSMV) is one of the most important for monitoring agrocenoses in Ukraine. The discovered dependence between the symptoms, physiological, biochemical reactions and the crop capacity of the infected plants which underwent the differences in air temperature may serve as the indirect diagnostic criterion [6, 7]. Primary non-specific plant response to biotic stress as well as the stress of not biotic origin is associated with lipid peroxidation (LPO), which intensity is measured, in particular, according to the contents of malon dialdehyde (MDA). In the process of lipid peroxidation the endogenous hydrogen peroxide (HP, $H_2O_2$) is produced. It plays an important indicating role in expressing the stability genes. Exogenous $H_2O_2$ is used to study the early responses of plants to stress and as an immune modulator [2, 4]. The investigation of relationships of stress reactions of virus infected plants may become part of ecosystem monitoring.

**The aim** of our work is to identify the relationship between primary and secondary reactions of WSMV – infected wheat plants to hypothermia and exogenous hydrogen peroxide.

**Materials and methods.** For this winter wheat "Pamyat'" was grown in controlled conditions. Three days seedlings were soaked in 100 mm $H_2O_2$ solution and subjected to high temperature tempering (TT) 4 degrees C. The duration of action in both cases was 1 hour. Two-weeks plants were mechanically inoculated by WSMV, some of them were kept for 15 min. in a refrigerator at the temperature damage (TD) – (- 3) degrees C. The MDA content was determined based on the reaction of 2-tiobarbiturova acid [1, 8] separately in the leaves and roots. The content of MDA was determined twice: 1 day after exposure to abiotic factors and at the end of the experiment (13 days plants), 14 days after infection (27 days plants). At the same time the growth response and antigen content of WSMV were estimated by enzyme immunoassay analysis (ELISA).

**Results and discussion.** Analysis of the primary reactions of plants at 1 day after exposure to both abiotic factors and WSMV showed that the level of MDA in leaves of wheat decreased compared with controls, after exposure to the virus and hypothermia that could temper the plants – by 54% (fig. 1).

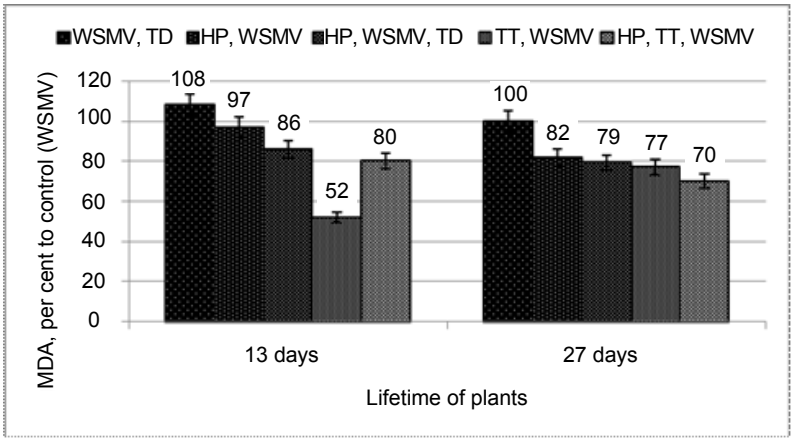


**Fig.1. Changes of PLO (MDA content, in % to control: WSMV) in the winter wheat plants in dynamic of infection process ($p<0.05$)**

In 10 days after exposure to abiotic factors MDA level came to normal. Two weeks after infection there was the increase in MDA level, which corresponded to increased concentrations of antigens WSMV (correlation coefficient 0.74): under the influence of the only biotic factor or in combination with negative temperature – by 22-84% (fig. 2).

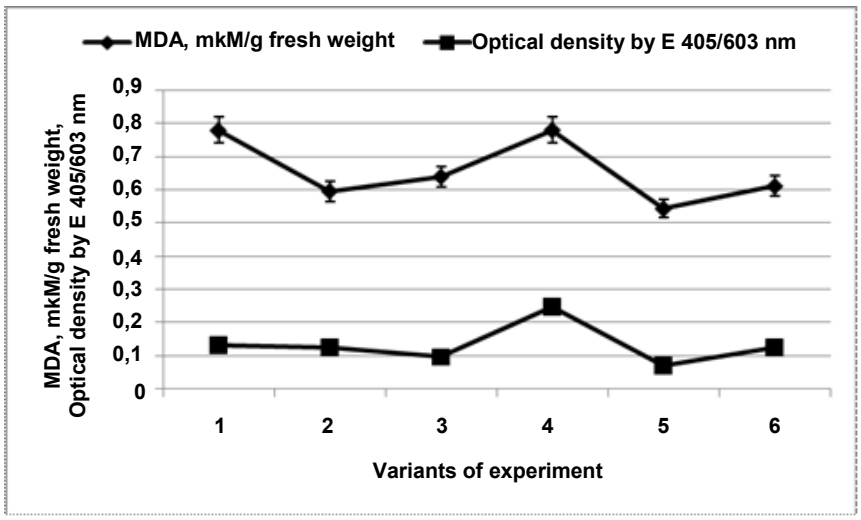


**Fig.2. A directcorrelationbetweenLPO (MDAcontent, mkM/gfreshweight); and results of ELISAinleaves of 27 days winter wheat plants (optical density E 405/603 nm). Correlation coefficient r = 0.74, p<0.05**

In infected plants, previously treated with peroxide or hardened with low positive temperatures, the intensity of lipid peroxidation was not significantly different from control. An inverse relationship between morphological metrics and the results of the ELISA was found (correlation coefficient -0.89) (fig.3)

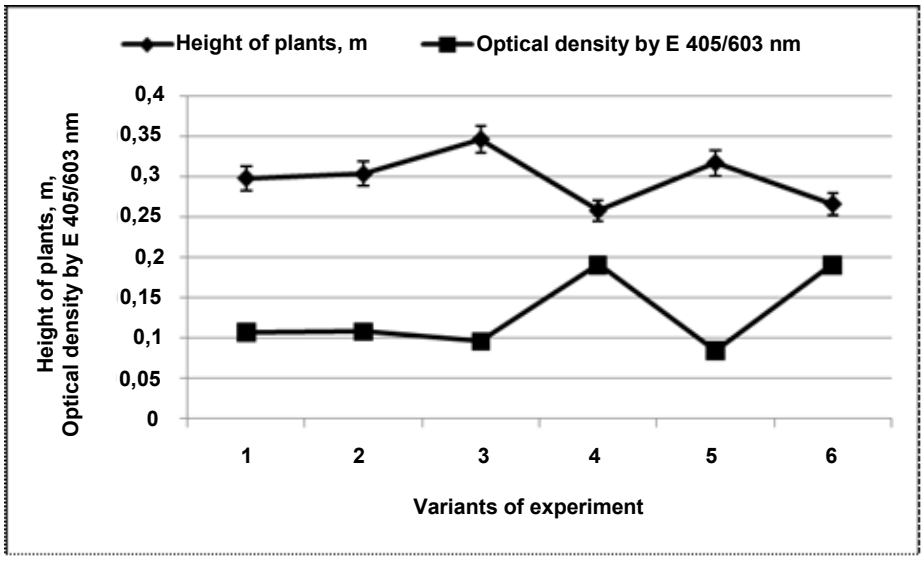


**Fig.3. An inverse relationship between morphological metrics in 27 days plants and the results of the ELISA in 27 days plants roots was found (correlation coefficient r = -0.89), p<0.05**

Revealed changes may indicate the development of immune reactions associated with antioxidant defense system of infected plants, pre-hardened with low positive temperature or treated with exogenous hydrogen peroxide. Our results and conclusions are coordinating with scientific sources [2–5].

**Conclusion.** The results can be used in ecosystem monitoring for predicting the probability of infection due to weather conditions and to develop safe methods of plant protection against pathogens of cereals.

1. Velikova V., Yordanov I., Edreva A. Oxidative stress and some antioxidant systems in acid rain treated bean plants. Protective role of exogenous polyamines // Plant Science. – 2000. – V. 151. – P. 59-66. 2. Бацманова Л.М. Активність супероксиддисмутази та каталази в листках проростків озимої пшениці за дії екзогенного пероксиду водню // Український біохімічний журнал, 2008. – т.80, №6. С.101–105. 3. Гарайда О., Кобилецька М., Терек О. Морфо-біохімічні показники проростків ZeamaysL. За дії саліцилової кислоти і фітопатогену // Вісник Львівського університету / Серія біологічна. – 2009. – Вип. 50. – С. 196–201. 4. Дмитрієв О.П., Кравчук Ж.М. Активні форми кисню та імунітет рослин // Цитологія і генетика, 2005. – №4. – С. 64–74. 5. Колупаев Ю.Е., Карпец Ю.В. Активные формы кислорода при адаптации растений к стрессовым температурам. – Физиология и биохимия культурных растений. – 2009. – Т.41. №2. – С.95 – 108. 6. Мищенко Л.Т., Решетник Г.В. Мониторинг и диагностика вирусных болезней озимой пшеницы в условиях меняющихся абиотических факторов Украины. Первая международная конференция по фитопатологии 25 – 27 сентября 2008. – Тбилиси. Грузия. С. 24. 7. Міщенко Л.Т., Решетнік Г.В., Тороп В.В. Комплексна діагностика симптомів багряних листків озимої пшениці. – Вісник аграрної науки, 2010. – С. 27–30. 8. Стальная И.Д., Гаришвили Т.Г. Метод определения малонового диальдегида с помощью тиобарбитуровой кислоты // Современные методы в биохимии / под ред. В.Н. Ореховича. – М.: Медицина, 1977. – С.66–68.

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# ОПРЕДЕЛЕНИЕ УРОВНЯ ПЕРЕДАЧИ ВИЧ ОТ МАТЕРИ К РЕБЕНКУ НА СОВРЕМЕННОМ ЭТАПЕ ЭПИДЕМИИ ВИЧ / СПИДА В УКРАИНЕ

***Проведен анализ распространенности ВИЧ-инфекции в Украине. Отмечен рост заболеваемости за последние 5 лет, что говорит о позднем обращении инфицированных. Обследование детей, рожденных от ВИЧ-инфицированных матерей и выявлена тенденция повторения эффективности АРТ в трансмисии вируса от матери к ребенку которая на данный момент составляет 4,7%.***

***The analysis of the prevalence of HIV infection in Ukraine are presented. Marked increase in cases over the past five years, indicating that the later handling infected. A study of babies born from HIV-infected mothers and a trend repeated ART effectiveness of transmission of the virus from mother to child is currently at 4.7% are presented.***

ВИЧ-инфекция, эпидемия которой началась в начале 80-х годов прошлого века, продолжает занимать ведущее место среди проблем здравоохранения и социального развития многих стран мира. Как отмечают специалисты Всемирной организации здравоохранения (ВОЗ) и Объединенной программы по ВИЧ/СПИДу Организации Объединенных Наций (ЮНЭЙДС) [1], ситуацию с ВИЧ-инфекцией/СПИДом в мире следует характеризовать как пандемию с катастрофическими демографическими последствиями для всех стран.

Анализ развития эпидемии ВИЧ-инфекции в Украине обосновал вывод о том, что она является одной из самых тяжелых и неконтролируемых среди стран Восточной Европы и Центральной Азии, особенно в сравнении с ситуацией в странах Западной и Центральной Европы [2,3].

За период 1987-2011 годов в Украине официально зарегистрировано 202 787 случаев ВИЧ-инфекции среди граждан Украины, в том числе 46 300 человек заболели СПИДом и 24 626 умерших от заболеваний, обусловленных СПИДом. Начавшись единичными случаями заболеваний, эпидемия ВИЧ/СПИДа набрала масштабных размеров: количество официально зарегистрированных случаев ВИЧ-инфекции ежегодно постоянно увеличивается. В 2011г. в стране официально зарегистрировано 21 177 новых случаев ВИЧ-инфекции (46,2 на 100 тыс. населения) – это самый высокий показатель за весь период наблюдения за ВИЧ-инфекцией в Украине с 1987г. [4].

Современное состояние развития эпидемического процесса ВИЧ-инфекции в стране характеризуется некоторым замедлением темпов роста новых случаев ВИЧ-инфекции. Об этом свидетельствует сравнение в промежутке 2001-2011 годов, а именно: темп прироста новых случаев ВИЧ-инфекции в 2001 году по сравнению с 2000 годом составил – 11%, в 2011 году по сравнению с 2010 годом – 3,6% (рисунок 1 , [5]).

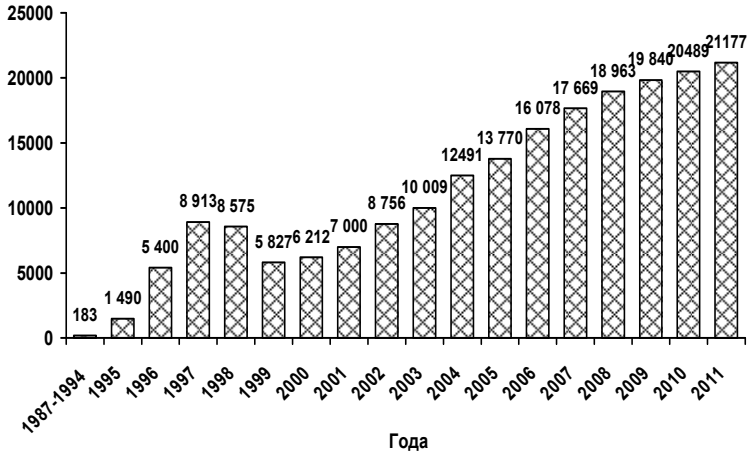


**Рисунок 1. Динамика официально зарегистрированных новых случаев ВИЧ-инфекции среди граждан Украины по годам за период 1987-2011 гг.**

Эпидемия концентрируется главным образом в городах: 77,1% новых случаев ВИЧ-инфекции в 2011 г. было зарегистрировано среди городского населения и 22,9% среди сельского.

В структуре ВИЧ-инфицированных преобладают лица в возрасте 25-49 лет, доля их постепенно растет (в 2007 г. – 62,8%; 2011г. – 66,3%). Вместе с тем, среди всех впервые зарегистрированных случаев ВИЧ-инфекции отмечается некоторое снижение числа заболеваний в возрастной группе 15-24 лет – с 15% в 2007 г. до 9% в 2011 г., что может быть следствием влияния профилактических программ среди молодежи. По состоянию на 01.01.2012 г., на диспансерном учете в учреждениях здравоохранения находилось 120 148 граждан Украины (264,3 на 100 тыс. нас.), Из них 18 751 – с диагнозом СПИД (41,2 на 100 тыс. нас.). Уровень заболеваемости СПИДом неуклонно растет, темп прироста в 2010 г. составил 57%, в 2009 г. – 1.8%. В последние годы среди впервые выявленных случаев ВИЧ-инфекции 44% – это лица с проявлениями СПИДа, свидетельствует о позднем обращении больных. Анализ заболеваемости СПИДом указывает на значительный рост числа больных, которое, по состоянию на 1.01.12 г., составляло 9 189 человек и среди них 5 745 (62,5%) больных туберкулезом. Перед здравоохранением встала новая проблема – ВИЧ-ассоциированным туберкулезом, который стал причиной смерти более чем 60% умерших от СПИДа. Одновременно с ростом заболеваемости СПИДом возросла смертность от болезней, обусловленных СПИДом: в 2011 году умерло 3736 больных (8,2 на 100 тыс. нас.), что на 20,6% больше, чем в предыдущий 2010 год (рис. 2). Начиная с 2007 г., наблюдается изменение основного пути передачи ВИЧ с парентерального, который доминировал на

протяжении 1995-2007 гг за счет потребителей инъекционных наркотиков. В 2008 г. отмечается преобладание полового пути: в 2011 г. число лиц, инфицированных половым путем, возросло до 49%, парентеральным, из-за введения инъекционных наркотиков, составило около 31% (рис. 3).

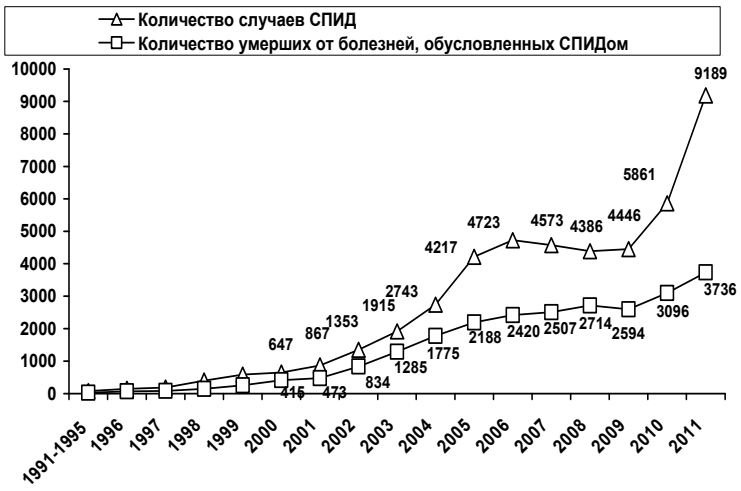


**Рисунок 2. Количество новых случаев СПИД и умерших от болезней, обусловленных СПИДом, среди граждан Украины в 1991-2011 гг.**

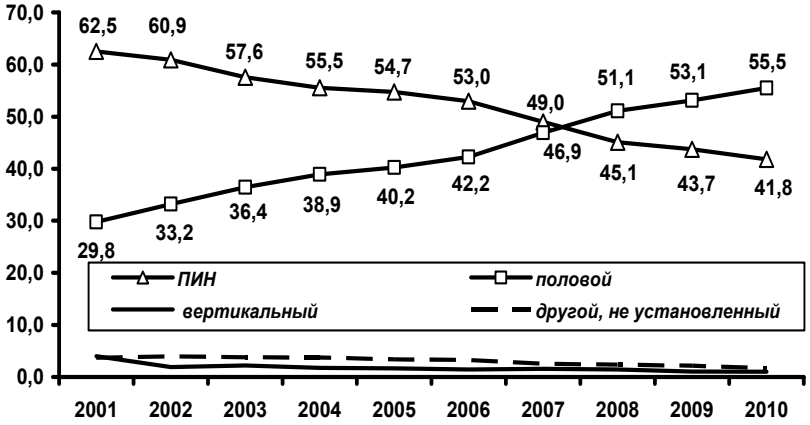


**Рисунок 3.Структура путей передачи ВИЧ в Украине,%**

Рост гетеросексуального пути передачи сопровождалось увеличением количества ВИЧ-инфицированных среди женщин детородного возраста (с 44,9% в 2009 г. до 45,5% в 2011г.), что повлекло постепенное увеличение количества детей, рожденных ВИЧ-инфицированными матерями. Риск передачи ВИЧ от матери к ребенку при отсутствии каких-либо профилактических мероприятий во время беременности и родов колеблется в пределах 20-45%, т.е. каждый 2-3 ребенок рождался с врожденной ВИЧ-инфекцией [6].

С 2000 г. при содействии и помощи ЮНИСЕФ в стране началась программа предупреждения передачи ВИЧ от матери к ребенку, дала первые положительные результаты уже через 2 года, когда частота передачи ВИЧ уменьшилась с 27,5% до 15%. Программа основана на внедрении антиретровирусной профилактики по короткой (тайской) схеме с 36 недели беременности. В последующие годы она была заменена более эффективной схемой профилактики.

В связи с особенностями диагностики ВИЧ-инфекции у детей, рожденных ВИЧ-инфицированными женщинами, все дети после рождения находятся под диспансерным наблюдением до момента окончательного установления ВИЧ-статуса (18 месяцев и старше). Несмотря на то, что все новорожденные сначала имеют положительный результат на антитела к ВИЧ за счет материнских антител, большинство из них являются ВИЧ-отрицательными, что подтверждается контрольными исследованиями в возрасте 18 месяцев. Дети, рожденные ВИЧ-инфицированными женщинами, у которых ВИЧ-статус не подтвердился в возрасте 18 месяцев и старше, снимаются с диспансерного учета.

И хотя в стране и наблюдается прогресс в профилактике передачи ВИЧ от матери к ребенку, общее количество детей с подтвержденным ВИЧ-позитивным статусом продолжает расти. На 01.01.2012 г. под наблюдением находятся 2 722 ребенка, у которых диагноз ВИЧ-инфекции подтвержден, в том числе 752 ребенка больны СПИДом, и 6 735 детей в стадии подтверждения диагноза ВИЧ-инфекции. Частота передачи ВИЧ при расчете через 18 мес. жизни, то есть за 2009 год, составляет 4,7% (рис. 4).

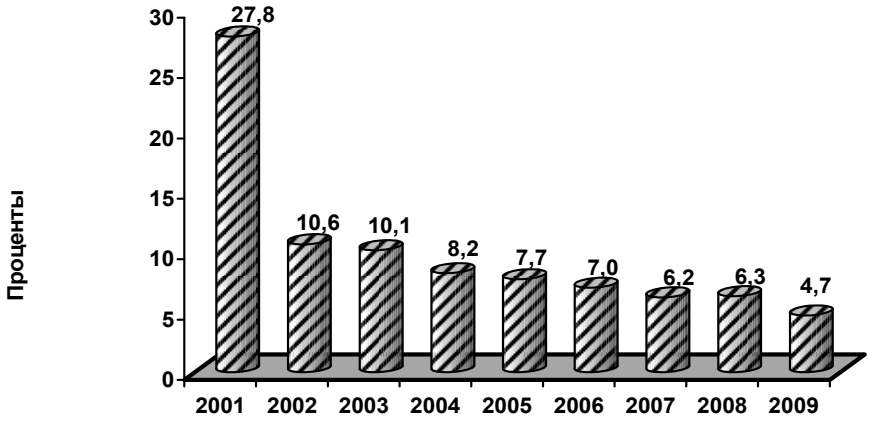


**Рисунок 4. Динамика показателячастоты передачи ВИЧ от матери к ребенку в Украине,%**

По отдельным регионам Украины показатели передачи ВИЧ колеблются от 0% в Житомирской до 16,7% в Хмельницкой области, как это показано на рисунке 5.

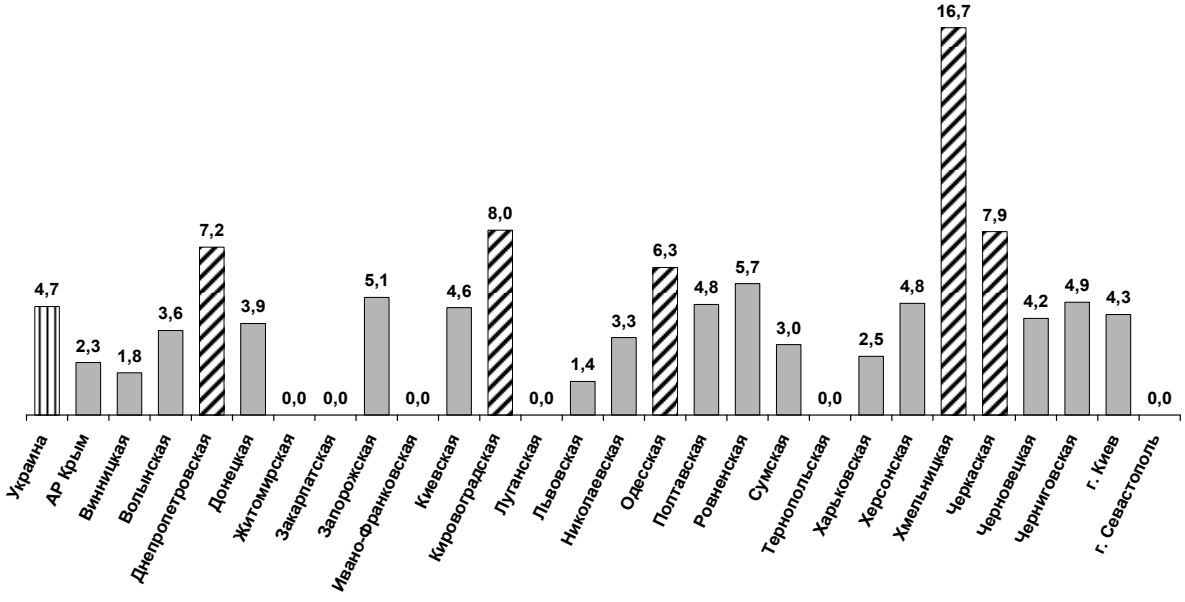


**Рисунок 5.Частота передачи ВИЧ от матери к ребенку в 2009 году по регионам Украины, %**

В исследованиях последних лет установлено, что введение ряда научно-обоснованных мер профилактики (специфическая химиопрофилактика АРВ-препаратами, выбор оптимальной тактики родов, отказ от грудного вскармливания) позволяет существенно снизить риск вертикальной трансмиссии до уровня менее 2%, или даже полностью предотвратить инфицирование детей. В 2012 г. ЮНЭЙДС обратился к мировому сообществу с призывом "НОЛЬ новых случаев инфицирования ВИЧ, НОЛЬ случаев смертей от СПИДа, НОЛЬ случаев дискриминации", что требует новых усилий по противодействию эпидемии ВИЧ-инфекции и в частности уменьшении до НУЛЯ частоты передачи ВИЧ от матери к ребенку [7].

Для оценки существующей в Украине системы профилактики вертикальной трансмиссии ВИЧ нами было проведено специальное исследование по определению показателя частоты передачи ВИЧ от матери к ребенку во время беременности и родов.

Для исследования использовались образцы цельной крови детей, рожденных ВИЧ-инфицированными матерями. Образцы крови исследовались на наличие провирусной ДНК ВИЧ-1 методом ПЦР с использованием тест-систем "АмплиСенс ® ДНК-ВИЧ-FRT" (АмплиСенс, Россия). Возраст детей, включенных в исследование, не превышал 18 месяцев. Показатель уровня вертикальной трансмиссии ВИЧ от матери к ребенку устанавливали путем определения процента детей, в образцах крови которых оказывалась провирусная ДНК ВИЧ-1, что свидетельствовало об их инфицировании от матери во время беременности или родов.

Были обследованы образцы крови 1600 детей, рожденных ВИЧ-инфицированными матерями, из них 760 детей обследованы дважды. Среди общего количества детей 1330 человек (83,13 ± 1,03%) получили полный курс специфической химиопрофилактики вертикальной трансмиссии ВИЧ (внутриутробном, в интра- и неонатальном периодах), 139 детей (8,69 ± 0,77%) получили неполный курс специфической химиопрофилактики АРВ-препаратами (только интра- и неонатальном периодах), 22 ребенка (1,38 ± 0,32%) вообще не получали специфической профилактики.

Провирусная ДНК ВИЧ-1 была обнаружена в образцах крови 49 детей, что составляет 3,06 ± 0,47% от общего количества обследованных лиц. Среди детей, получив-

ших полный курс специфической профилактики вертикальной трансмиссии, положительные результаты исследования на наличие ДНК ВИЧ-1 в крови были установлены у 17 человек (1,28 ± 0,95%). В группе детей, которые получили неполный курс специфической профилактики этот показатель составлял 14,38 ± 4,45%, а в группе детей, которые вообще не получали специфической профилактики, показатель частоты передачи ВИЧ от матери к ребенку был высоким и составлял 36,36 ± 10,25%.

**Выводы**. Проведенные нами исследования свидетельствуют о том, что низкий уровень инфицирования ВИЧ детей, получивших полный курс специфической профилактики, подтверждает высокую эффективность введенных в Украине профилактических мероприятий по предупреждению вертикальной трансмиссии ВИЧ от матери к ребенку. В то же время, стоит отметить, что согласно полученных нами данных, дети, которые не получали специфическую профилактику вертикальной трансмиссии ВИЧ или получили неполный курс профилактики, составляют основную группу риска инфицирования ВИЧ от матери во время беременности и родов.

1. Развитие эпидемии СПИДа. Декабрь 2009 г.95 стр. 2.Онищенко Г.Г. Противодействие эпидемии ВИЧ/СПИДа в Восточной Европе и Центральной Азии. //Журн. микробиол. – 2009. – №1. – С.16-21. 3. Дементьева Л.А., Голиусов А.Т. Особеннсти эпидемии ВИЧ-инфекции в Восточной Европе и Центральной Азии на современном этапе. //Журн. микробиол. – 2010. – №2. – С.32-34. 4. ВІЛ-інфекція в Україні. Інформаційний бюлетень. // К., 2011. – №37. – 89 стор. 5. Гармонізований звіт України про досягнутий прогрес у здійсненні національних заходів у відповідь на епідемію СНІДу. 2012 рік.// Київ, 2012. – 240 стор. 6. European Collaborative Study. Mother-to-child transmission of HIV infection in the era of highly active antiretroviral therapy. //Clin. Infect. Dis. – 2005. – Vol.50. – P.458-465. 7. United to the tnd AIDS: achieving the targets the 2011 Political Declaration. United Natiob. General Assembly. A/66/757. 2 April 2012.

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# ВИРУС ЯМЧАТОСТИ ДРЕВЕСИНЫ ЯБЛОНИ В МОЛДОВЕ

***В коллекционном саду яблони Института садоводства, виноградарства и пищевых технологий в Кишиневе (Республика Молдова) отобран изолят вируса ямчатости древесины и успешно перенесен на растения индикатора Nicotianaoccidentalis 37B. Приготовлена поликлональная антисыворотка для идентификации вируса в различных тканях пораженных деревьев иммунологическими методами ELISA и ISEM. Изучена распространенность этого вируса в насаждениях яблони Республики. Методом ОТ-ПЦР отобраны изоляты для последующего проведения сиквенса и филогенетического анализа.***

***In apples' collection garden of Instetute of Horticulture, Viticulture and food technologies in Kishinev (Republic of Moldova)*** **Apple** ***Stem Pitting Virus were isolated and successfully transmitted on the indicator-plants Nicotiana occidentalis 37B. The polyclonal antiserum was obtained for identification of the virus in different tissues of affected trees by serological methods ELISA and ISEM. The spread of the virus among apple's plantations of Republic were observed. According to the RT-PCR data virus isolates for further sequence and phylogenetic analysis were selected.***

Ямчатость древесины яблони (ASPV) впервые описана в 1954 году в США (8), а вирусная природа доказана в 1956 году после передачи возбудителя прививкой на здоровые растения древесных индикаторов (5). В настоящее время заболевание встречается во всех странах, где культивируют яблоню. До недавнего времени этиология ASPV не была изучена. К вирусным заболеваниям его относили на основании вызываемых им симптомов и диагностику проводили в полевых опытах на древесных индикаторах. В 1986 году возбудитель ASPV был успешно перенесен на растения индикатора Nicotianaoccidentalis 37B (9). В 1990 году японским исследователям удалось повторить перенос ASPV с деревьев яблони с симптомами ямчатости на табак N. occidentalis 37B, приготовить антисворотку к нему и на основании результатов последующих исследований показать, что ВЯДЯ является новым видом вируса с удлиненной морфологией частиц (6).

Согласно последней классификации, ASPV отнесен к роду *Foveavirus* семейства *Flexiviridae.*

В Молдове ASPV впервые обнаружен и описан в 1969 году (2). Установлено, что после вируса хлоротической пятнистости листьев яблони ASPV наиболее часто встречаемый на семечковых культурах (1).

В 1994 году в Институте плодоводства в Кишиневе был выделен изолят ASPV из яблони, перенесен на индикатор N.occidentalis 37B и приготовлена антисыворотка с титром 1/2000. Однако приготовленные на ее основе диагностикумы выявляли исследуемый вирус методом иммуноферментного анализа только в тканях лепестков, ограничивая сроки тестирования растительных образцов.

Исходя из сказанного нам предстояло:

- приготовить антисыворотку для ELISA диагностики исследуемого вируса в течение года;
- изучить распространенность ASPV в насаждениях яблони Молдовы;
- методом полимеразной цепной реакции отобрать изоляты ASPV для последующего сиквенирования и филогенетического анализа.

Для получения диагностической антисывортки к ВЯДЯ нами были протестированы сортообразцы яблони из коллекционного сада института садоводства на зараженность исследуемым вирусом. Тестирование проводили методом иммуносорбентной электронной микроскопии (ISEM) антисывороткой любезно предоставленной доктором Янасе (Япония) в период цветения с использованием тканей лепестков. Отбор изолята проводился по результатам количества выявляемых вирусных частиц в сканируемых препаратах. По этим параметрам было отобрано 5 образцов 1-9-63, 1-15-33, 1-17-13, 1-17-63 и 3-32-11. Полученные результаты были подтверждены повторным тестированием этих же сортообразцов с использованием, кроме лепестков, экстракта коры молодых побегов и отрастающих листьев. Для механического переноса ВЯДЯ на индикатор N.occidentalis 37B были использованы ткани лепестков. Растения индикатора заражали в стадии 3-4 настоящих листьев при температуре 22-25°С. Экстрагирующие смеси готовили на основе 3-х буферных смесей (таблица 1). Перед заражением листья индикатора опудривали карборундом.

***Таблица*** **1. Влияние буферных растворов на перенос ВЯДЯ на индикатор N/occidentyalis 37B**

| № п/п | Наименование буферных смесей | Исследуемые изоляты | | | | |
|---|---|---|---|---|---|---|
| | | 1-9-63 | 1-15-33 | 1-17-13 | 1-17-63 | 3-32-11 |
| 1 | 0,05М pH 7,5 HEPES | - | - | - | - | - |
| 2 | 0,02М pH 7,8 Tris-HCl | - | - | - | - | - |
| 3 | 0,05 М pH 7,8 K/Na phospat buffer +0,1% sodium sulfat, +0,1% sodium sulfit, + 0,1% thioglicerol | - | - | - | - | + |

Из таблицы видно, что положительный результат был получен только в варианте № 3 с использованием в качестве источника инфекции лепестки образца 3-32-11. На 27-29 день после заражения на отрастающих листьях некоторых растений, появились симптомы в виде просветления жилок 2 и 3 порядка (Рис.1), Результаты же переносов с использованием инокулюмов остальных образцов оказались отрицательными, в том числе изолята 3-32-11 в вариантах 1 и 2.

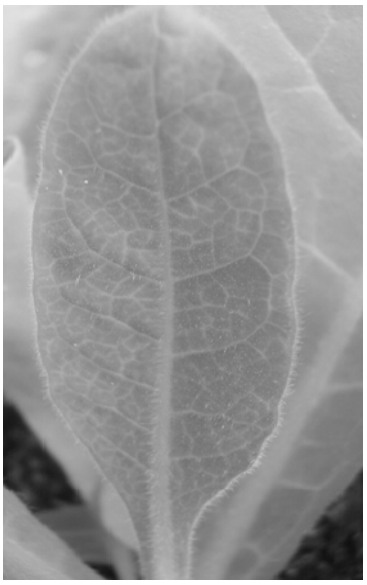

**Рис 1. Просветление жилок на индикаторе N.occidentalis 37B вызываемое ASPV**

Листья с симптомами просветления жилок были использованы для последующего пассажа с целью размножения вируса в тканях индикатора. Пассаж вируса проводили с помощью фосфатного буфера без добавок, а инфицированные растения индикатора выращивали в условиях короткого дня с целью максимального накопления вируса в зараженных тканях. На 12-14 день после заражения на отрастающих молодых листьях появлялись описанные выше симптомы, которые и использовали для очистки ВЯДЯ. Свежесорванные листья с симптомами гомогенизировали в в 0,02М pH 7,0 Na фосфатном буфере с добавлением 0,1% тиоглицерола в соотношении 1:4 и процеживали через 4 слоя марли. Полученный экстракт центрифугировали 10 мин при 5 000g. В надосадочную жидкость (НЖ) добавляли гидратированный фосфат кальция при осторожном перемешивании в течение 30 мин. Затем суспензию центрифугировали 10 мин при 5 000g. НЖ центрифугировали над 5 мл 20% сахарозы в течение 90 мин при 120 000g в роторе Ti 45 BECKMAN. Осадок суспендировали в 0,01М pH 8,0 фосфатном буфере содержащем 0,5% TritonX-100 и центрифугировали 10 мин при 5 000g. НЖ наслаивали на 30% сахарозную подушку и центрифугировали 90 мин при 100 000 g в том же роторе. Полученный осадок суспендировали в 0,01М боратном-HCl буфере содержащем 0,5% мочевины в течение ночи. Затем суспензию центрифугировали в градиенте плотности сахарозы (10-40) 180 мин при 80 000 g в роторе SW 28 BECKMAN. Вирусную зону диализовали против фосфатного буфера в течение ночи. Чистоту препарата проверяли с помощью спектрофотометра "SPECOL 1500" (Рис. 2).

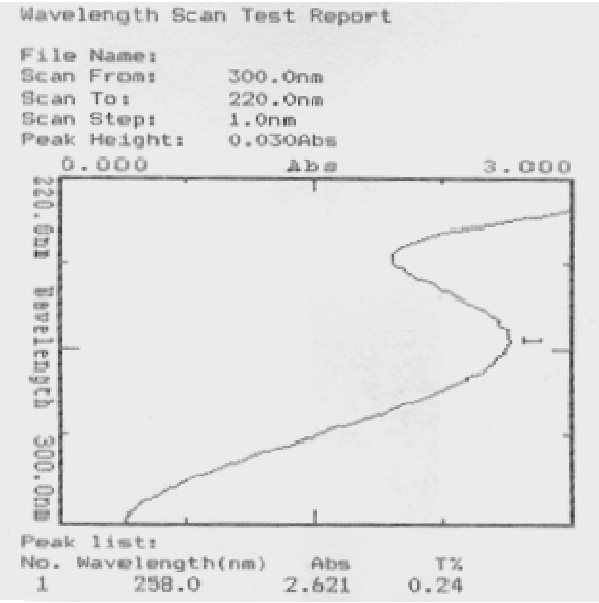


**Рис 2. Спектрограмма очищенного препарата ASPV**

Для получения антисыворотки был проведен короткий цикл иммунизаций кроликов – подкожно с полным адъювантом Фрейнда, внутримышечно с неполным адъювантом Фрейнда и внутривенно. Активность антител в крови подопытных кроликов определяли методом ISEM. На 22 день после первой иммунизации титр антисыворотки составлял 1:2000. Через 4 недели активность антисыворотки стала падать. После двух реиммунизаций активность антител была восстановлена и сохранялась до 127 дня после первой иммунизации.

Диагностические наборы для ELISA готовили из сывороток, полученных на 90 и более дней после первой иммунизации. Фракцию иммуноглобулина IgG выделяли методом аффинной хроматографии с использованием протеина A-sepharosaCL 4B фирмы "SERVA" (3). Коньюгат готовили по описанному в литературе методу (4) с использованием в качестве маркера щелочную фосфатазу. В результате проведенных исследований было установлено, что для надежного выявления ВЯДЯ необходимо микроплаты сенсибилизировать иммуноглобулином в концентрации 0,75 mkg/ml. Оптимальное разведение коньюгата должно составлять 1:1000 – 1:1500.

В 2011 году нами были проведены исследования по изучению распространенности ВЯДЯ в насаждениях яблони в Молдове. Общая площадь обследованных сортов составила 75 га. В процессе обследований рандомизировано отбирали пробы для лабораторного исследования. Тестирование проводили методом ISEM с использованием антисыворотки нашего производства. Всего было тестировано 805 образцов, из которых 122 были заражены ВЯДЯ, что составляет 14,1%. Установлено также, что ВЯДЯ в пораженных образцах встречается вместе с двумя другими латентными вирусами – хлоротической пятнистости листьев (CLSV) и бороздчатости древесины (ASGV). Все три вируса в природе не имеют переносчиков и распространяются только при их вегетативном размножении. Как показали результаты, исследуемый вирус, как и 2 остальных вируса, наиболее часто выявлялись в деревьях возрастом 20 и более лет, чем в молодых насаждениях, что может быть связано с общей тенденцией закладки садов оздоровленным посадочным материалом.

Проведены исследования по изучению выделенных в процессе обследования изолятов ASPV методом полимеразной цепной реакции (ПЦР).

Тотальную РНК выделяли с помощью специального набора фирмы "Quagen". Для проведения реакции использовали коммерческий сет фирмы "InvitrogenSupperStrip" с парой праймеров, подобранных Menzel (7). Полученные в результате ПЦР фрагменты были фракционированы методом электрофореза в 2% агарозном геле. Результаты электрофореза визуализировали и регистрировали в ультрафиолете (Рис. 3).

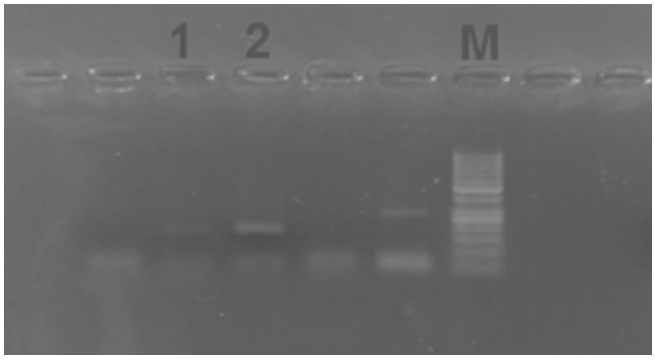


**Рис.3. Электрофореграмма продуктов ОТ-ПЦР: М-маркер молекулярных масс 100 б/п; 1, 2-образца ASPV**

Выделенные в результате РТ-ПЦР фрагменты будут использованы в дальнейших исследованиях с целью сиквинирования и проведения филогенетического анализа родства изолятов ASPV.

1. Бивол, Т.Ф. Латентные вирусы яблони и их изучение в Молдавской ССР. Вирусные болезни плодово-ягодных культур и винограда в Молдавии. Выпуск 1. Кишинев, с. 32-65. 1973. 2. Вердеревская, Т. Д., Рудь, Г.Я. Вирусные заболевания плодовых культур и винограда и пути решения проблемы создания свободных от вирусов насаждений. Доклады семинара ФАО стран Средиземноморья по защите плодовых и винограда от вредителей и болезней. – М., с. 11-18. 1969. 3. Clark M.F., Adams A.N. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. Vol.34, No.3, p.475-483. 1977. 4. Clark M.F., Bar-Joseph M. Enzyme immunosorbent assay in plant virology. In: Methods in Virology (Eds. K.Maramorosch, A.Kaprowski), N.Y.:Academic Press. p 51-85. 1984. 5. Guengerich, H.W., and Millikan, D.F. Transmission of the stem pitting factor in apple. Plant Dis. Rep. 40:934-938. 1956. 6. Koganezawa, H., and Yanase, H. A New Type of Elongated Virus Isolated from Apple Trees Containing the Stem Pitting Agent. Plant Dis. 74:610-614. 1990. 7. Menzel, W., Jelkmann, W., Mais, E. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant m RNA as internal control. Journal of Virological Methods 99:81-92. 2002. 8. Smith, W.W. Occurrence of "stem pitting" and necrosis in some body stocks of apple trees. Proc. Am. Soc. Hortic. Sci. 63:101-113. 1954. 9. van der Meer, F.A. Observations on the etiology of some virus diseases of apple and pear // Acta Hortic. 193;73-74.1986.

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# ВЛИЯНИЕ ВИРУСНОЙ ИНФЕКЦИИ НА ЦИТО-ГИСТОЛОГИЧЕСКОЕ И ФУНКЦИОНАЛЬНОЕ СОСТОЯНИЕ ХМЕЛЯ (*HUMULUS LUPULUS* L.)

***В Украине на плантациях хмеля Житомирской области методом ОТ-ПЦР выявлены: Hop latent virus, Cucumber mosaic virus и Apple mosaic virus; установлено, что на начальных стадиях вирусной инфекции в листьях хмеля изменяется направление переноса и распределение фенольных веществ, а их общий пул увеличивается в 8-9 раз; в инфицированных вирусами листьях хмеля выявлено повышение интенсивности автофлуоресценции метаболитов в голубом (460-500 нм) и желто-зеленом (546-575 нм) спектрах; данный эффект может быть применен в экспресс-диагностике растений на вирусоносительство.***

***Using RT-PCR there are detected: Hop latent virus, Cucumber mosaic virus and Apple mosaic virus on the hop plantations of Zhitomir region in Ukraine; the direction of transfer and distribution of phenolic substances are changed on the initial stages of viral infection and their general pool is raised in 8-9 times; in hop leaves, affected with virus, the increasing of autofluorescence intensity of metabolic products in blue (460-500 nm) and yellow-green (546-575 nm) spectrums is determined; it can be used as an express method of plant viral diseases.***

**Введение.** Вирусные болезни хмеля обыкновенного (*Humulus lupulus* L.) существенно снижают синтез α- и β-кислот, ароматических и горьких веществ, которые являются основными коммерческими продуктами культуры [6]. Достоверно известно 20 вирусов, поражающих растения хмеля обыкновенного [2]. Наиболее вредоносными среди них являются вирусы мозаики хмеля (ВМХ), латентный (ЛВХ) и американский латентный (АЛВХ) вирусы хмеля, а также вирус мозаики яблони (ВМЯ) [2,5]. При системном пораженнии растений ВМХ содержание α- и β-кислот в шишках хмеля уменьшается почти в четыре раза [5]. При заражении вирусная инфекция достаточно быстро распространяется по растительному организму, приобретая системный характер. В условиях вертикального переноса вирусные частицы в неинокулированных листьях вначале обнаруживаются в трахеидах мелких проводящих пучков, откуда они через клетки обкладки попадают в палисадную паренхиму, клетки которой на первых стадиях патогенеза поражаются более интенсивно [7]. Деструктивные процессы в тканях вегетативных и генеративных органов растений сопровождаются существенным увеличением синтеза вторичных метаболитов в протопластах и клеточных стенках [10]. Одним из наиболее изученных механизмов защиты растений от различных патогенов является синтез производных фенилпропаноидов [11]. К данной группе относятся сесквитерпеноиды, изофлавоноиды, дигидрофенантрены, способные нарушать целостность цитоплазматических мембран фитопатогенных организмов [13]. Барьерные свойства клеткам обеспечивают связанные с клеточными стенками кислые пероксидазы, которые активно участвуют в синтезе компонентов лигнина и суберина [1]. Особая роль в формировании конституционной устойчивости растений отводится оксидазам, гидролитическим ферментам, хитиназам и β-1,3-глюканазам [12]. Ответной реакцией растительного организма на вирусную инфекцию является синтез салициловой кислоты (СК) – эндогенного соединения фенольной природы. Последняя способна угнетать процесс репликации и транслокации вирусов в тканях растений [14]. В конъюгированной форме она образовывает метилсалицилат – летучее соединение, повышающее экспрессию генов PR-1 белков (pathogenesis-related) и содействующее развитию реакции системной устойчивость растений к вирусам [10]. Центром синтеза и накопления СК являются хлоропласты, откуда она легко транспортируется по флоэме. В гептане и подкисленном этаноле СК под действием ультрафиолетового света (УФ) сильно флуоресцирует с максимумом эмиссии в длине волны 480 нм, а в этилатенатрия – 420 нм [3], что позволяет обнаруживать это фенольное вещество в тканях растений. Способность к автофлуоресценции в живых клетках растений имеют также целлюлоза (420-430 нм), фенолы (450-460 нм), NAD(P)H (460 нм), терпены, некоторые алкалоиды и флавоноиды (470-525нм), флавины (520 нм), каротиноиды (540-550 нм), антоцианины, азулины (600-630 нм) и хлорофиллы (675-680 нм) [9,15]. При изучении вирусного патогенеза автофлуоресценция эндогенных соединений в тканях растений имеет определенные преимущества перед исследованиями фиксированного и гомогенизированного материала, поскольку данный подход позволяет наблюдать центры синтеза, локализации и транспорта вторичных метаболитов, а также структуру и физиологическое состояние живых клеток.

**Целью** наших исследований было изучение цито-гистологического и общего функционального состояния растений хмеля обыкновенного, инфицированных вирусами, по показателям автофлуоресценции внутриклеточных метаболитов.

**Объекты и методы исследований.** Исследования проводили на растениях хмеля обыкновенного, отобранных на плантациях Олевского района Житомирской области, которые были заложены в 2004-2007 годах на безвирусной основе с применением технологии *in vitro*. Листья хмеля сортов "Национальный" и "Заграва" отбирали со среднего и нижнего ярусов с учетом степени проявления патогенеза: мозаичности, точечных и межжилковых хлорозов, некрозов.

Тотальную РНК экстрагировали из растительного материала реагентами комплекта "РИБО-сорб" AmpliSens® согласно стандартному протоколу. Реакцию обратной транскрипции проводили с использованием коммерческого набора "Реверта", следуя рекомендациям производителя. Диагностику и идентификацию вирусов, поражающих хмель, проводили методом полимеразной цепной реакции (ПЦР) с использованием диагностических тест-систем, разработанных в лаборатории фитовирусологии и биотехнологии НУБиП Украины. Объектами исследований были латентный вирус хмеля, вирус огуречной мозаики и вирус мозаики яблони. ПЦР проводили в объеме реакционной смеси – 25 мкл (реагенты AmpliSens®, Россия) с использованием 5 пкмоль каждой пары праймеров. Амплификацию выполняли на оборудовании Applied Biosystems 2400. Условия проведения реакции: начальная денатурация 94°С – 5 мин, далее 35 циклов в следующем режиме: денатурация 94°С – 30 с, ренатурация (гибридизация) 55°С – 30 с, элонгация 72°С – 30 с. Заключительный цикл – финальная элонгация 72°С – 7 мин. Электрофорез продуктов амплификации проводили в 2%-ном агарозном геле в ТАЕ-буфере с использованием маркера молекулярных масс GeneRule™ 100 bp DNA Ladder и УФ визуализацией с бромистым этидием.

Анатомическое строение и структурные изменения листьев изучали на нативных и постоянных микропрепаратах толщиной 8-10 мкм. Повторность в цитологических и гистохимических исследованиях 10-ти кратная. Растительные ткани обрабатывали фиксатором Чемберлена (60 % этиловый спирт, формалин и ледяная уксусная кислота – 90:5:5; продолжительность фиксации – 24 ч). Люминесцентный анализ листьев проводили на свежесрезанном материале без предварительной фиксации и окрашивания флюорохромами. Автофлуоресценцию тканей изучали с использованием узкополосных оптических фильтров (Carl Zeiss), способных пропускать свет в диапазоне длин волн: 420-470 нм, 460-500 нм, 505-555 нм, 546-575 нм, 567-647 нм и 660-690 нм. Растительные ткани исследовали на эпилюминесцентном микроскопе Carl Zeiss AxioScope A-1. Фотодокументацию и обработку экспериментальных данных выполняли в специализированной программе для анализа цифровых изображений – Image-Pro Premier 9.0 (*Evaluation version*).

**Результаты и их обсуждение.** Наиболее выраженными симптомами вирусного повреждения хмеля (*Humulus lupulus*) являются проявления на его листьях различных типов хлорозов и межжилковой мозаики. Патогенная депигментация листьев, как правило, связана со структурными изменениями хлоропластов, разрушением молекул хлорофилла и других фотосистем. Рисунок и динамика проявления мозаичности, хлоротических изменений имеет определенную функциональную связь с особенностями васкулярного транспорта вирусов в тканях листьев (рис. 1 А-Г).

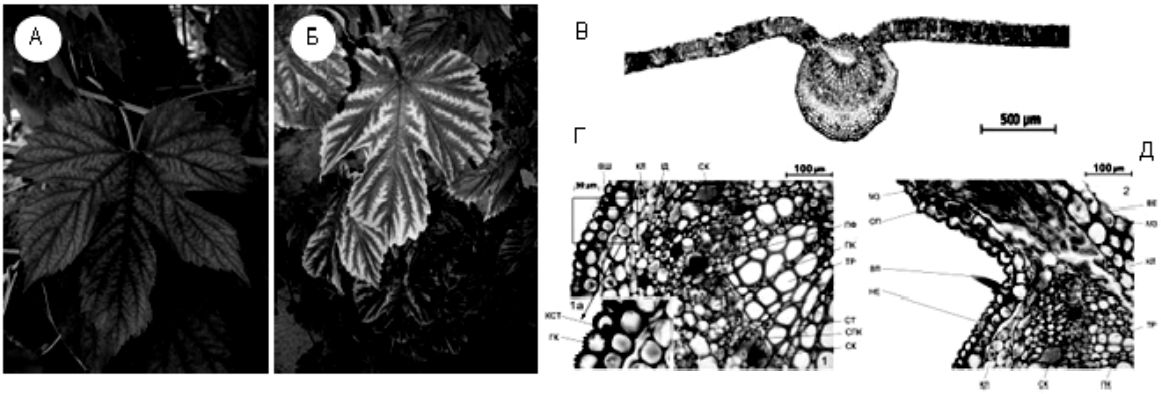


**Рис. 1. Листья хмеля обыкновенного:** начальная (А) и завершающая (Б) стадии межжилкового вирусного хлороза; В – поперечный срез листовой пластинки; Г, Д – строение центральной жилки листа (поперечный срез, фрагменты); вш – верхний слой кутикулы; ве – верхний эпидермис; гк – гребенчатая кутикула; вл – волосок; не – нижний эпидермис; мз – мезофилл; оп – обкладка проводящего пучка; кл – колленхима; ск – секреторный канал; тр – трахея; пф – паренхима флоэмы; пк – паренхима ксилемы; ст – ситовидная трубка; спк – спутничная клетка; ід – идиобласт; кст – клеточная стенка

Ранее нами уже было показано [5], что пассивная транслокация вирусных частиц происходит по ксилеме к конечным трахеидам мелких проводящих пучков. Далее они поступают в апопласт и с током воды перемещаются преимущественно через клетки обкладки в палисадную паренхиму. Рассмотренные особенности транспорта вирусов в тканях листа объясняют характер развития вирусной мозаики и межжилкового хлороза. Как правило, первые симптомы проявляются между жилками второго и третьего порядков (рис. 1 А). В случаях прогрессирующей вирусной болезни хлороз листьев хмеля достигает значительной контрастности до полного нарушения характерной для данного вида пигментации (рис. 1 Б).

Для идентификации и подтверждения вирусной природы мозаики листьев хмеля нами были проведены диагностические исследования с применением мультиплексной ОТ-ПЦР. В результате были получены ампликоны размером – 427 п.н., 157 п.н., 109 п.н., которые находятся в оптимальном диапазоне, отвечающем участкам генома *Hop latent virus* (HLV), *Cucumber mosaic virus* (CMV), *Apple mosaic virus* (ApMV) соответственно. Таким образом, в исследуемых нами образцах (рис. 2, пробы 3-4) было подтверждено присутствие вирусной РНК. Интересным фактом является ПЦР идентификация в листьях хмеля вируса огуречной мозаики (ВОМ), который в растениях этого вида выявлен в Украине впервые.

Растения хмеля обыкновенного характеризуются активным образованием вторичных метаболитов [4]. Значительная их часть синтезируется и накапливается в хлоропластах. В нормальных условиях в хлоропластах мезофилла здоровых листьев хмеля происходит активный синтез лейкоантоцианидинов и флавонол-гликозидов. Часть их переносится через плазмалемму и клеточную стенку в межклеточное пространство, поступает в мелкие проводящие пучки, и далее, через центральную жилку транспортируется в стебель и другие органы растения. Многие фенольные соединения, синтезируемые в листьях по шикиматному и ацетатно-малонатному пути, обладают способностью к автофлуоресценции, что позволяет выявлять центры их синтеза и накопления, наблюдать движение этих веществ по тканям растения, а также исследовать некоторые процессы биохимической трансформации продуктов метаболизма в процессе пластического обмена.

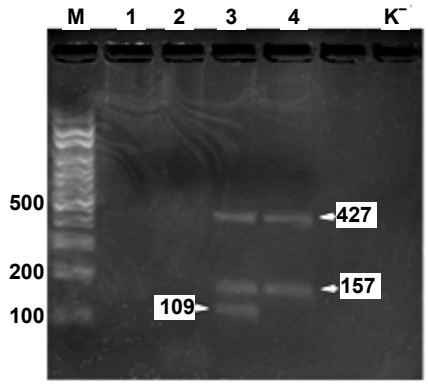


**Рис. 2. Електрофоретическое разделение продуктов амплификации ОТ-ПЦР из листьев хмеля:**
M – маркер молекулярных масс GeneRule™ 100 bp DNA Ladder; 1, 2, 4 – сорт "Национальный"; 3 – сорт "Заграва"; K⁻ – отрицательный контроль

К таким веществам относятся лейкоантоцианидины, флавоноиды, флавонол-гликозиды, фенолкарбоновые кислоты (оксибензойная, салициловая, транскоричная), которых особенно много в листьях хмеля обыкновенного [4]. Результаты люминесцентной микроскопии свежесрезанных листьев хмеля показали, что в синем спектре (420-460 нм) наибольший квантовый выход наблюдался в клетках верхнего и нижнего эпидермисов (рис. 3 а-б), а также в цистолитах и идиобластах. Максимальное поглощение ультрафиолетового света (340-390 нм) было выявлено нами в клетках столбчатой и губчатой паренхимы. Данный спектр поглощения соответствует максимуму (380 нм), который характерен для флавонол-гликозидов. Установленный факт подтверждает данные относительно более высокого содержания этих метаболитов в мезофилле листьев, по сравнению со стеблем и корнем [4]. У растений, пораженных вирусами, наибольшая интенсивность флуоресценции в листьях отмечалась в голубом (460 – 500 нм) и желто-зеленом (546-575 нм) спектрах (рис. 3 б, г).

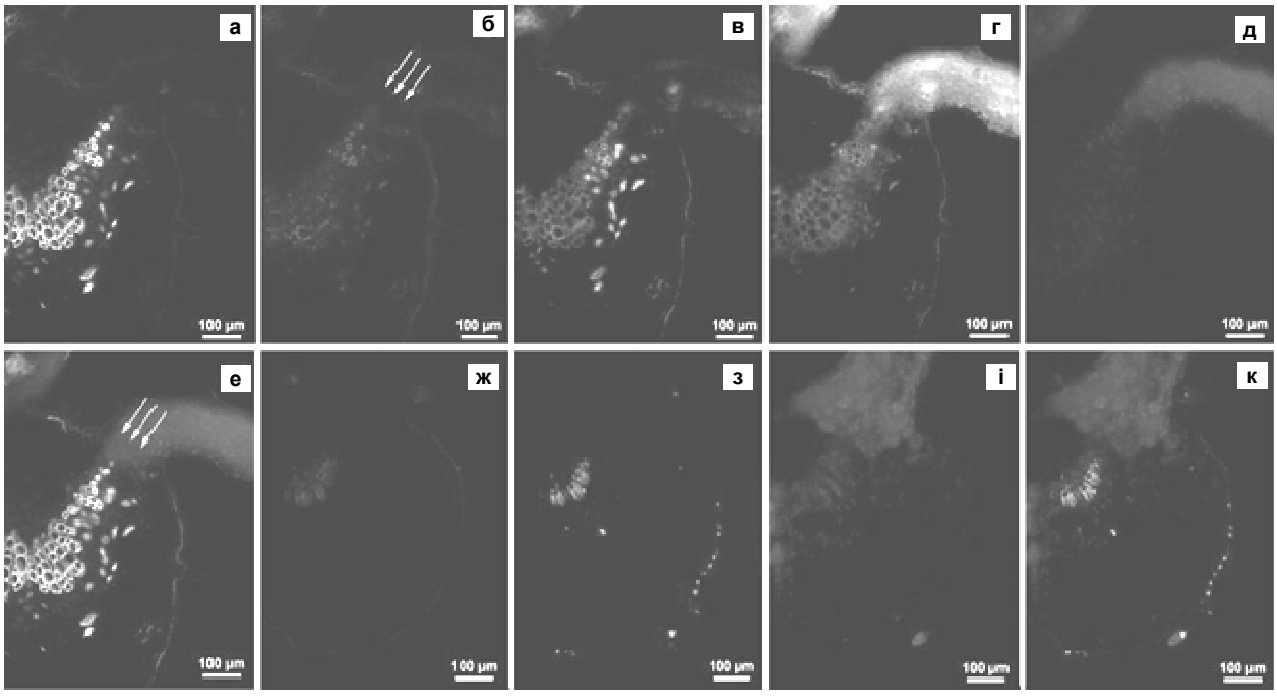


**Рис. 3. Автофлуоресценция веществ в жилке II-порядка и тканях листовой пластинки с симптомами вирусной мозаики (а-е) и бессимптомного (ж-к) листа хмеля (*Humulus lupulus*):**
а, ж – световая эмиссия в спектре – 420-470 нм; б– 460-500 нм; в, з – 505-555 нм; г – 546-575 нм; д, і – 640-690 нм; е, к – комбинированное изображение тканей в оптических фильтрах 430 нм и 680 нм; (стрелками указано направление уменьшения градиента концентрации биогенных люминофоров)

По характерному для фенолов и СК спектру эмиссии (460-480 нм) было установлено, что их общее содержание и распределение в тканях растения зависит от наличия патогенов вирусной природы. В эпидермисе листьев здорового растения содержание фенолов было в 2-3 раза больше, чем в листьях с признаками вирусной мозаики (рис. 4 А-В). Тем не менее, синтез веществ фенольной природы и СК на начальных стадиях вирусной инфекции в тканях столбчатой и губчатой паренхимы возрастал в 6-8 раз (рис. 4 Б). Инфекционный процесс часто сопровождается увеличением содержания в клетках мезофилла терпенов и флавоноидов, способных к флуоресценции с наибольшим квантовым выходом в зеленом спектре (505-555 нм). Под действием вирусов их концентрация в цитоплазме клеток мезофилла быстро растет (рис. 4 В).

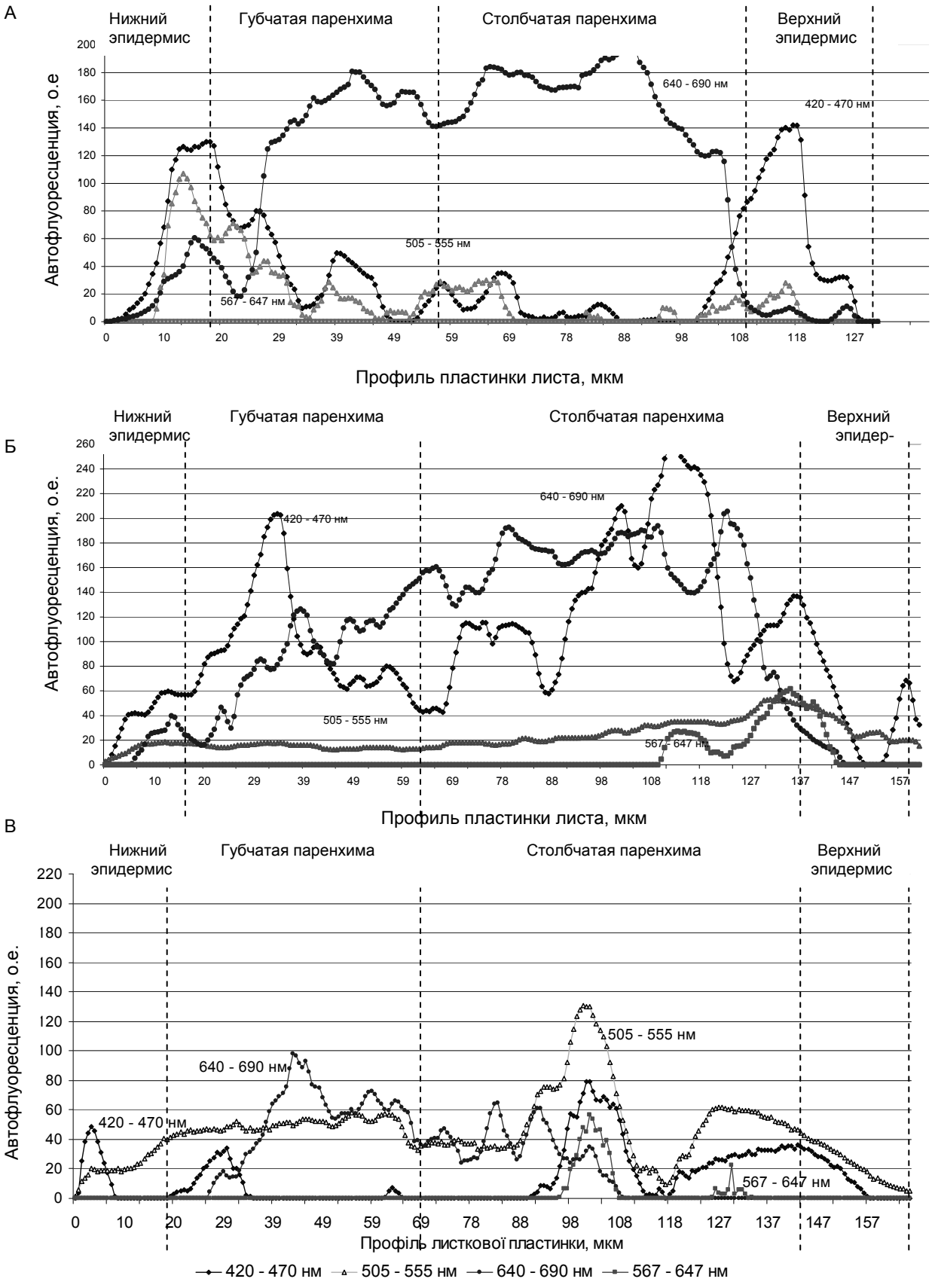


**Рис. 4. Линейный профиль автофлуоресценции метаболитов в тканях листьев хмеля (*Humulus lupulus*) (поперечный срез): бессимптомный лист (А); начальная (Б) и завершающая (В) стадии межжилковой вирусной мозаики**

На завершающих стадиях развития вирусной межжилковой мозаики суммарное содержание терпенов и флавоноидов в верхнем эпидермисе повышается в 3 раза, а в клетках столбчатой паренхимы в 9 раз (табл. 1). Флуоресценция хлорофилла по отношению к контролю, наоборот, вначале несколько увеличивается (на 15-18%), а затемсущественно уменьшается (до 70%).

***Таблица* 1. Влияние вирусной инфекции на динамику содержания в листьях хмеля (*Humulus lupulus*) биогенных люминофоров**

| Стадия патологических изменений | Структурные элементы листа | Содержание биогенных люминофоров по отношению к контролю, % | | | |
|---|---|---|---|---|---|
| | | 420-470 нм | 505-555 нм | 567-647 нм | 640-690 нм |
| Начальная стадия межжилковой мозаики | ВЭ* | 114,6 | 443,6 | 200,1 | 95,7 |
| | СП | **813,0** (инд) | 336,2 | **1500,0** (инд) | 118,0 |
| | ГП | 309,1 | 72,4 | – | 99,1 |
| | НЭ | 55,5 | 27,6 | – | 55,1 |
| | Всего | 340,4 | 164,1 | **> 1700** | 91,2 |
| Межжилковая вирусная мозаика | ВЭ | – | 294,2 | – | 0 |
| | СП | 236,5 | **911,3** | 324,6 | 80,8 |
| | ГП | 120,1 | 221,7 | – | 127,8 |
| | НЭ | 23,8 | 66,0 | – | 85,9 |
| | Всего | 77,5 | 298,1 | 324,6 | 91,4 |
| Межжилковая вирусная мозаика с признаками некрозов | ВЭ | 26,0 | 440,2 | – | 0 |
| | СП | 120,8 | **920,3** | **611,5** | 23,0 |
| | ГП | 24,4 | 318,1 | – | 38,6 |
| | НЭ | 13,7 | 27,3 | – | 0 |
| | Всего | 48,3 | 320,4 | **611,5** | 27,2 |

* ВЭ – верхний эпидермис; СП – столбчатая паренхима; ГП – губчатая паренхима; НЭ – нижний эпидермис; инд – потенциальный индикатор вирусной инфекции

Заметно ослабляется также флуоресценция тканей листьев в синем спектре. Этот эффект может быть обусловлен общим уменьшением концентрации фенолов и фенолкарбоновых кислот в листьях, их биохимической трансформацией в соединения, которые не способны к световой эмиссии в исходных условиях. На основании полученных нами данных по автофлуоресценции продуктов метаболизма складывается представление о том, что в системе распределения фенольных соединений в тканях листьев хмеля существует определенная градиентная структурированность, которая может быть представлена следующей схемой (рис. 5).

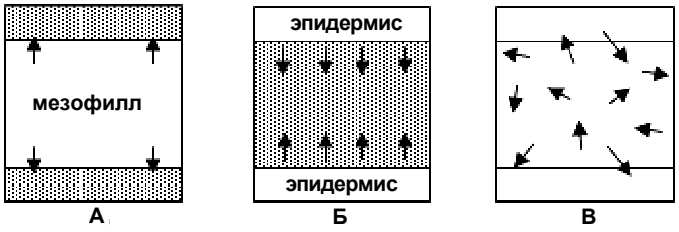


**Рис. 5. Влияние вирусной инфекции на распределение и направление переноса фенольных соединений в листьях хмеля обыкновенного: здоровый лист (А); начальная (Б) и завершающая (В) стадии межжилковой вирусной мозаики**

До проникновения вирусной инфекции в растительный организм в сформировавшихся листьях наблюдается тенденция к переносу фенольных веществ от центров их синтеза в покровные ткани (клетки верхнего и нижнего эпидермисов) (рис. 5 А). На начальных стадиях вироза синтез фенолов в мезофилле листьев значительно активизируется, но их перенос к клеткам эпидермиса приостанавливается (рис. 5 Б). Данный физиологический процесс, вероятно, связан с неспецифической защитной реакцией растительного организма, которая сопровождается снижением проницаемости плазмалеммы для фенольных веществ, активизацией синтеза и отложения компонентов лигнина, β -(1→3 )-глюканов (каллозы) и других биополимеров в клеточных стенках [10]. На завершающей стадии проявления вирусной мозаики общее содержание фенолов, способных к флуоресценции, уменьшается почти вдвое, а их распределение в тканях листьев теряет системность (рис. 5 В) и приобретает диффузный характер.

**Выводы**

1. Впервые в Украине установлен факт поражения хмеля (*Humulus lupulus*) вирусом огуречной мозаики.

2. На исследованных плантациях Житомирской области в растениях хмеля методом ОТ-ПЦР выявлена смешанная вирусная инфекция, которая представлена *Hop latent virus*, *Apple mosaic virus* и *Cucumber mosaic virus*.

3. Установлено, что в условиях смешанной вирусной инфекции на начальных стадиях межжилковой мозаики в листьях хмеля увеличивается общий пул и направление межклеточного транспорта фенольных соединений.

4. В листьях хмеля, инфицированных вирусами, выявлено значительное повышение интенсивности автофлуоресценции веществ в голубом (460-500 нм) и желто-зеленом (546-575 нм) спектрах; на начальных стадиях вирусного заболевания содержание продуктов вторичного метаболизма с максимальной световой эмиссией в синем (420-470 нм) и желто-оранжевом (567-647 нм) спектрах увеличивается по отношению к контролю, соответственно в 8 и 15 раз; данный эффект может быть применен в экспресс-диагностике вирусных инфекций растений.

1. Андреева В.А. Фермент пероксидаза: участие в защитном механизме растений. – М., 1988. 2. Бойко А.Л. Вирусы и вирусные заболевания хмеля и розы эфиромасличной. – К., 1976. 3. Красовицкий Б.М., Болотин В.М., Органические люминофоры / Под ред. Б.М. Красовицко-

го. – М., 1984. 4. Ляшенко Н.И., Михайлов Н.Г., Рудык Р.И. Физиология и биохимия хмеля / Под ред. Н.И. Ляшенко – Житомир, 2004. 5. Мельничук М.Д. Біотехнологічний процес одержання рослин хмелю на безвірусній основі / М.Д. Мельничук, О.Є. Кріпкий, А.Л. Бойко // Доповіді НАН України. – 1996. – № 9. – С. 139-141. 6. Мельничук М.Д. Проблема карлавірусної інфекції хмелю в Україні // Науковий вісник НАУ. – 1998. – № 4. – С. 28-33. 7. Мельничук М.Д. Реакция сверхчувствительности растений при инфицировании фитовирусами // Бюл. ін-ту с.-г. мікробіол. – 2000. – №6. – С.25-28. 8. Московець С.М., Бойко А.Л. Вірусні захворювання хмелю та заходи боротьби із ними / За ред. С.М. Московця. – К., 1970. 9. Рощина В.В., Рощина В.Д.Выделительная функция высших растений / Под ред. В.В. Рощиной. – М., 1989. 10. Шакирова Ф.М. Неспецифическая устойчивость растений к стрессовым факторам и ее регуляция. – Уфа, 2001. 11. Dyxon R.A. Palva N.L. Stress-induced phenylpropanoid metabolism. Plant cell. – 1995. – №7. – P. 1085-1097. 12. Hammond-Kosack K.E, Gones JDG Resistance gene dependent plant defence responses. Plant Cell. – 1996. – №8. – P. 1773-1791. 13. Mauch-Mani B. Metraux J.P. Salicylic acid and systemic acquired resistance to pathogen attack. Annals Bot. – 1998. – №82.– P. 535-540. 14. Murphy A.M., Chivasa S., Singh D.P., Carr J. Salicylic acid-induced resistance to viruses and other pathogens: a parting of the ways? Trend Plant Sci. – 1999. – №4. – P. 155-160. 15. Roshchina V.V. Fluorescing world of plant secreting cells. – Jersey (USA), 2008.

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# ИНГИБИРОВАНИЕ ИНФЕКЦИОННОСТИ ВИРУСА ТАБАЧНОЙ МОЗАИКИ В ПРИСУТСТВИИ ГЛЮКАНА *GANODERMA ADSPERSUM* (SCHULAR DONK) В ИЗОЛИРОВАННЫХ ПРОТОПЛАСТАХ ТАБАКА

***Изучено влияние полисахарида, экстрагированного с мицелия гриба Ganoderma adspersum,на репродукцию вируса табачной мозаики в протопластах табака. Показано, что гликан ингибирует репродукцию незначительно. Активность препарата обусловлена, очевидно, его действием на процессы адсорбции и проникновение вируса в клетку. Гликан не вызывает агрегации вирионов, что показано при центрифугировании комплекса вирус-полисахарид в градиенте плотности сахарозы, но приводит к деградации вирионов. Данный факт свидетельствует о том, что в общей антивирусной активности глюкана Ganoderma adspersum, важное место имеет его влияние на внеклеточный вирус.***

***The effect of polysaccharide extracted from the mycelium of Ganoderma adspersum, on the tobacco mosaic virus reproduction in isolated cells of tobacco has been investigated. It is shown that glucan insignificantly inhibits a virus reproduction in isolated cells, and its activity is conditioned mostly by the influence on adsorption processes and virus entry into a cell. Glucan does not cause aggregation of virions, that was shown after centrifugation of the complex virus-polysaccharide in sucrose density gradient but it can cause of virions degradation. This fact confirms that in the total anti-virus activity of glucan Ganoderma adspersum important place has an effect of polysaccharide on an extracellular virus.***

Известно, что высшие базидиальные грибы продуцируют биологически активные полисахариды с уникальной структурой [9]. Полисахаридные препараты, экстрагированные с мицелия, плодовых тел. и культуральной жидкости грибов, имеют свойства ингибировать развитие опухолей, стимулировать иммунные реакции, в некоторых обнаружены антивирусные и антимикробные свойства [1, 6]. Среди грибов-продуцентов биологически активных препаратов особое место принадлежит грибам семейства *Ganoderma*. Издавна их плодовые тела использовали японские императоры как пищевые добавки для улучшения своего здоровья и долголетия. Препараты, изготовленные на основе грибов, сегодня широко используются в фармакологи как общеукрепляющие и противоопухолевые средства.

В последнее время для грибных полисахаридов установлена способность ингибировать развитие вирусных инфекций растений [5]. В частности глюкуроноксиломаннан *Tremellamesenterica* и глюканы *Ganoderma adspersum,* могут индуцировать устойчивость сверхчувствительных растений к вирусу табачной мозаики (ВТМ), а так же ингибировать его в у чувствительных растений [2, 3].

Целью данной работы было детальное изучение влияния полисахаридов базидиальных грибов на разные этапы репродукции вируса в изолированных протопластах табака [7].

**Материалы и методы.** *Объекты исследований:* полисахаридный препарат, выделенный путем водной экстракции с мицелия *Ganoderma adspersum* (штамм II, полученный с коллекции Хайфського университета, Израиль), вирус табачной мозаики (ВТМ, штамм $U_1$, полученный путем дифференционного центрифугирования) протопласты, изолированные с листьев табака *Nicotianatobaccum*, сорта Иммунный 580, что мутантный по N-гену [8].

*Методы исследований*. Протопласты получали с листьев табака возрастом 6-8 недель одноступенчатым методом [8] с помощью 2% целюлазы (Onozuka R-10) и 0,1% мацерозима R-10, растворенных средой для культивирования состава: $KH_2PO_4$ – 27,2 мг/л $KNO_3$ – 101,0 мг/л, $CaCl_2*2H_2O$ – 1480,0 мг/л $MgSO_4*7H_2O$ – 246.0 мг/л KI – 0,16 мг/л $CuSO_4*5H_2O$ – 0,025 мг/л, 0,5 М манитол (pH 5,8). Листья растений табака стерилизовали 7% раствором "Доместос" 20 мин., 4 раза промывали стерильной дистиллированной водой после чего снимали нижний эпидермис и помещали на стерильный раствор ферментов на 2 часа. После экстракции протопласты отделяли от остатков ткани центрифугированием (100 g, 2 мин) и отмывали от ферментов средой для отмывки состава: 0,5 М манитол, 5 мМ $CaCl_2$ и $KH_2PO_4$. Протопласты, что получили, культивировали в среде CPW.

Концентрацию клеток в 1 мл суспензии определяли, подсчитывая их в камере Горяева используя 0,2 %-й раствор метиленового синего.

Протопласты инфицировали с помощью поликатиона poly-D-lisine (~120000). Раствор поликатиона (1 мкг/мл) добавляли к суспензии ВТМ (1 мкг/мл), выдерживали 10 мин., вносили к изолированным клеткам и инкубировали 10 мин., периодически перемешивая. Вирус, который не адсорбировался на клетках, отмывали средой для отмывки 3 раза.

Для проверки влияния глюкана на адсорбцию вируса и его активность в клетке полисахаридный препарат в концентрациях 200 и 500 мкг/мл вносили в среду для культивирования протопластов и к суспензии вирус-поликатион. Были проверены следующие варианты: 1 – препарат вносили во среду для культивирования, 2 – препарат добавляли к инокулят, 3 – препарат добавляли к инокулят и к среде для культивирования. Отрицательным контролем служили не инфицированные протопласты, положительным – инфицированные протопласты, не обработанные полисахаридом.

Во время культивирования протопластов в динамике проверяли количество антигена ВТМ методом иммуноферментного анализа (ИФА). Образцы протопластов анализировали через 24, 48 и 72 ч. после инфицирования протопластов и обработки их препаратом.

Для изучения влияния полисахарида на репродукцию ВТМ в изолированных клетках глюкан (200,

500 мкг/мл) добавляли в культуральную среду к протопластам, что были получены с инфицированных растений табака. Контролем служили инфицированные клетки без полисахарида. Во время культивирования инфекционность ВТМ в протопластах определяли, инокулируя листья *Datura stramonium* образцами клеток отобранными через 2, 6 и 24 часов после глюкана. Клетки отмывали от манитола 0,01 М фосфатным буфером и замораживали для разрушения ткани и экстрагирования вируса. Полученный гомогенат размораживали и центрифугировали (8000 g, 10 мин.). Супернатант использовали для определения антигена методом ИФА, а также инфекционности вируса на растениях-индикаторах.

С целью исследования влияния глюкана на структуру вирионов ВТМ готовили смесь вируса и полисахарида в конечных концентрациях 2 и 5 мг/мл, соответственно. Выдерживали 30 мин и центрифугировали в градиенте плотности сахарозы (10-45 %, 22 тыс. об/мин., центрифуга Bekmen 40), после чего определяли положение опалесцирующей зоны, что содержала вирус, просматривая пробирки в ультрафиолете. В качестве контроля использовали смесь вируса с 0,01 М фосфатным буфером в идентичной концентрации. С каждой пробирки были отобраны образцы для электронно-микроскопических исследований.

**Результаты и обсуждения.** Раньше нами было показано, что полисахарид, полученный с мицелия гриба *Ganoderma adspersum* путем водной экстракции и очищенный от примесей белка, является гетерогенным препаратом и состоит из 3-х компонентов с молекулярным весом 20, 48 и 95 кДа [4]. Каждый из них ингибирует инфекционность ВТМ в чувствительных и сверхчувствительных растениях табака. Механизм антивирусной активности полисахаридного препарата заключается в активации защитных реакций у сверхчувствительных растений и влиянии на этапы репродукции у чувствительных. Показано, что активность этого препарата в сверхчувствительном сорте табака подобна к ГКМ *Tremella mesenterica* [3], который ингибирует этапы синтеза белков в клетке. Между тем, данные препараты ингибируют репродукцию ВТМ в сверхчувствительных растениях табака.

Для более детального изучения действия полисахарида на репродукцию ВТМ в данной работе мы использовали систему изолированных протопластов. Исследовали влияние препарата на процессы проникновения и репликации вируса в клетках. Для этого полисахарид добавляли к инокуляту и в культуральную среду в разных вариантах. Установлено, что при различных способах применения глюкана титр вируса был сниженным, по сравнению с контролем (Рис. 1), причем, с повышением концентрации полисахарида до 500 мкг / мл его активность растет.

Таким образом, глюкан *G. adspersum* может ингибировать репродукцию вируса в изолированных клетках. Причем, самый высокий уровень угнетения наблюдался при его применении одновременно с вирусом. Данный факт может свидетельствовать о том, что исследуемый полисахарид, очевидно, влияет не непосредственно на репродукцию вируса в клетке, а на процесс его адсорбции и проникновения.

Полученные результаты были подтверждены при обработке полисахаридом протопластов, что были изолированы из растений табака инфицированных ВТМ. В данном случае проникновение вируса в клетку уже произошло и снижение титра вируса под влиянием препарата возможно только при условии ингибирования его репродукции. В опыте использовали табак, чувствительный к ВТМ. Протопласты получали из листьев растений на 12 сутки после инокуляции их вирусом.

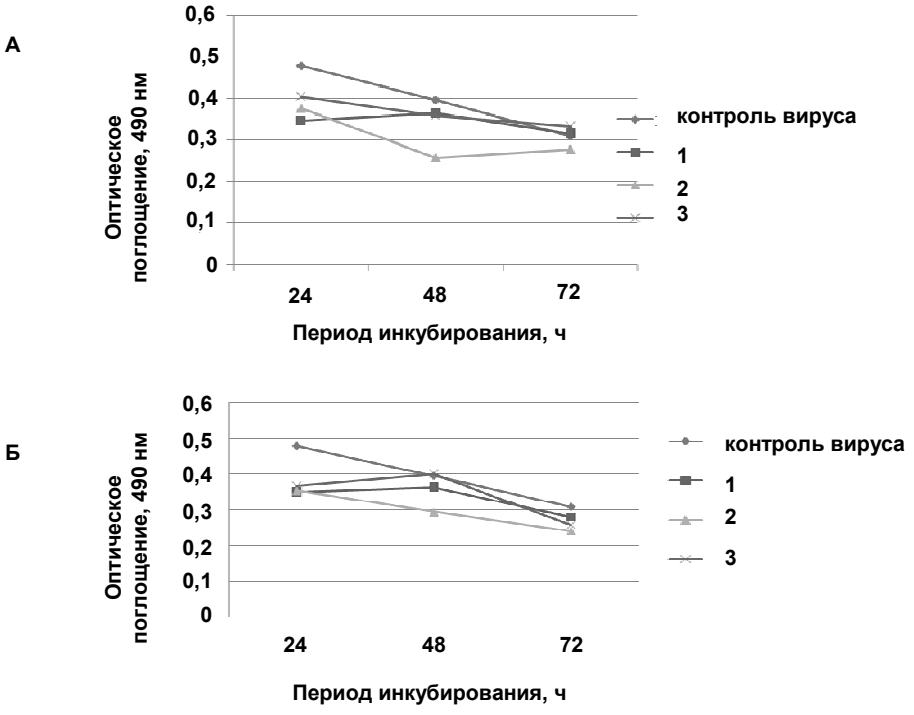


**Рис 1. Влияние глюкана на репродукцию ВТМ в изолированных протопластах (концентрация полисахарида 200 мкг / мл (А) и 500 мкг / мл (Б))**
**Препарат добавляли:**
1-к инокуляту и в культуральную среду; 2-к инокуляту; 3-в культуральную среду

При добавлении полисахаридного препарата в инокулят была отмечена высокая антивирусная активность, которая проявлялась через 24 и 48 ч после инфицирования. При обработке протопластов до инфи-

цирования и добавлении препарата в культуральную среду, ингибирование вируса было низким, а при введении глюкана только в культуральную среду – уровень угнетения вируса занимал промежуточное положение.

Установлено, что глюкан в концентрации 500 мкг/мл через два часа после обработки протопластов снижал титр антигена ВТМ в 4 раза по сравнению с контролем, чего не наблюдалось через 6 часов после обработки. Через 24 часа инкубации снижение титра вируса во всех исследуемых вариантах было незначительным по сравнению с контролем (Табл.1).

***Таблица* 1. Титр антигена ВТМ в изолированных протопластах табака, обработанных глюканом *G. adspersum***

| Период действия препарата, ч | Глюкан, 200 мкг/мл | Глюкан, 500 мкг/мл | Контроль |
|---|---|---|---|
| 2 | 1:640 | 1:160 | 1:640 |
| 6 | 1:320 | 1:160 | 1:160 |
| 24 | 1:80 | 1:80 | 1:160 |

При исследовании инфекционности ВТМ, экстрагированного из протопластов, что были обработаны глюканом, показано, что инфекционный титр вируса был снижен на 10-15% во всех исследуемых вариантах (Рис. 2).

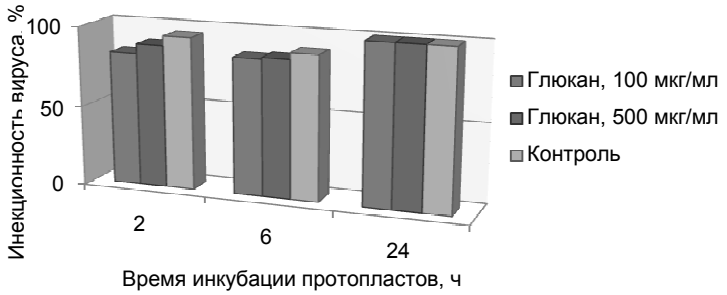


**Рис. 2. Влияние глюкана на инфекционность ВТМ в изолированых протопластах табака**

Глюкан не имел заметного цитотоксического влияния ни в одной из исследуемых концентраций (Рис. 3), что исключает возможность снижения биологической активности ВТМ за счет нарушения гомеостаза клеток.

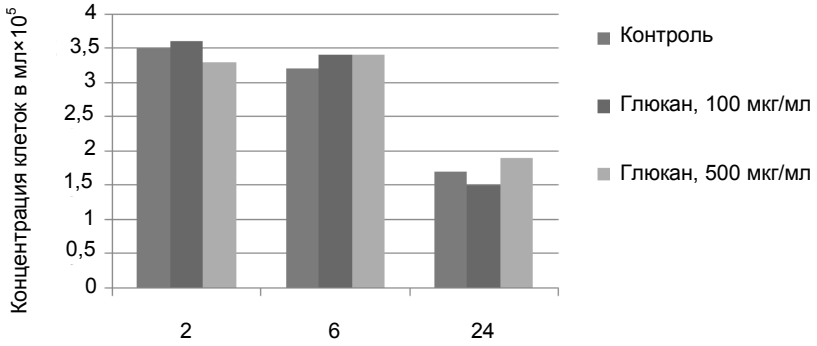


**Рис. 3. Влияниеразных концентрацій на жизнедеятельность протопластов табака инфицированных ВТМ**

Таким образом, полисахарид, полученный из мицелия *G.adspersum*, в системе изолированных протопластов имеет незначительное влияние на репродукцию вируса, по сравнению с активностью в системе растительных тканей [6]. Поскольку обработка протопластов глюканом не влияет на их жизнеспособность, можно сделать вывод, что антивирусная активность данного препарата не вызвана токсическим эффектом или его способностью активировать процессы, приводящие к гибели клетки.

Одним из возможных влияний полисахарида на внеклеточный вирус может быть его действие на структуру вирионов. Для проверки такой возможности нами было проведено центрифугирования суспензии вирус-полисахарид в градиенте плотности сахарозы. Такой прием позволяет разделить взаимодействующие компоненты в зависимости от их молекулярной массы. Нами было установлено, что как в контрольном варианте, где полисахарид отсутствовал так и в опытном (с полисахаридом) опалесцирующая зона вируса имела одинаковое положение. При центрифугировании вируса с полисахаридом было отмечено опалесцирующую зону в градиенте плотности, которая соответствовала 5% концентрации сахарозы, чего не было в контроле. Данная зона имела светло-коричневый окрас, характерный для глюкана, в частности, его низкомолекулярной фракции [3]. При исследовании каждой зоны в электронном микроскопе видно, что в контроле в поле зрения микроскопа наблюдались единичные вирионы ВТМ, которые имели целостную структуру (Рис. 4, А). В опытном варианте, вирионы ВТМ находились в зоне 15%, которая отвечала вирусу в контроле. При исследовании образцов, отобранных из варианта обработанного глюканом, вирионы были разрушены. В поле зрения встречались частицы, имеющие размер менее 100 нм (Рис. 4 Б, В).

Таким образом, исходя из результатов электронно-микроскопических исследований, можно сделать вывод, что исследуемый глюкан при взаимодействии с вирусом способен вызвать деградацию вирионов. Полученные результаты могут служить объяснением высокой антивирусной активности этого препарата при добавлении его к вирусу и инокуляции растений дурмана [3].

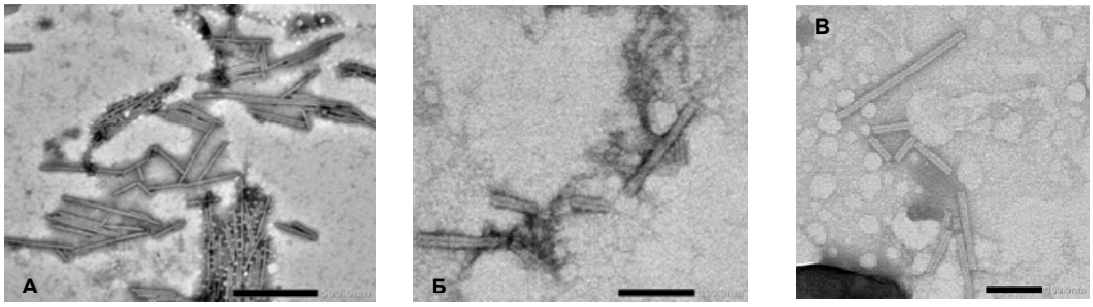


**Рис. 4. Электронная микроскопия ВТМ, полученного с опалесцирующей зоны, которая сформировалась после центрифугирования в градиенте плотности сахарозы:**
А –вирус после центрифугирования без глюкана, Б, В – вирус после центрифугирования в присутствии глюкана

Поскольку из предыдущих исследований известно, что глюкан *G.adspersum* подавляет инфекционность ВТМ в системе растительных тканей, а также способен активировать защитные механизмы растений [2, 3], полученные результаты могут указывать на то, что ингибирование репродукции вируса не является основанием антивирусной активности этого препарата. Частичное снижение репродукции вируса в протопластах, очевидно, происходит только за счет влияния препарата на вирус в клетке, а именно на его структуру. Поскольку антивирусная активность препарата выше при его добавлении к инокулят чем при одновременном введении в инокулят и культуральную среду, можно сделать вывод о возможном влиянии глюкана на гомеостаз растительных клеток и, как результат, невосприимчивость растения к вирусним инфекциям. Однако данное предположение нуждается в детальных исследованиях.

1. Даниляк М.І. Лікарські гриби. Медичне застосування та проблеми біотехнології. /М.І. Даниляк, С.В. Решетніков // Київ 1996. – 64 с. 2. Коваленко О.Г. Вплив глюкороноксиломанану Tremellamesentericа-Ritz.: Fr. (Basidiomycota) на стійкість рослин до вірусу тютюнової мозаїки / О.Г. Коваленко, О.М. Поліщук // Мікробіол. журн. – 2009. – 71, № 1. – С. 50-56. 3. Коваленко О.Г. Глікани вищого базидіального гриба Ganodermaadspersum (Schulzer) Donk: отримання та антифітовірусна активність / О.Г. Коваленко, О.М. Поліщук, С.П. Вассер // Біотехнологія. – 2010. – Т.3. – №5. – С.83-91. 4. Поліщук О.М. Глікани базидіоміцетів як інгібітори ВТМ-інфекції та індуктори вірусостійкості рослин // О.М.Поліщук. // Автореферат на дисертацію. – 2011 р. – 23 с. 5. HuiS. Alectin with my celiadifferentiation and antiphytovirus activities from theedible mushroom Agrocybeaegerita/ S. Hui, C. G. Zhao, X. Tong // Journalof-BiochemistryandMolecularBiology. – 2003. – V. 36. – P.214–222. 6. Lindequist U. The pharmacological potential of mushrooms / U. Lindequist, T.H.J. Niedermeyer, W. Julich // eCAM. – 2005. – V .2. – P. 285–299. 7. Otsuki Y. Tobacco mosaic virus multiplication and expression of the N-genein necrotic responding tobacco varieties / Y. Otsuki, T. Shimomura, I. Takebe // Virology. – 1972. – V. 50. – P. 45–50. 8. Plant cell culture: a practical approach [edited by R.A. Dixon].– IRL Press Limited. – Oxford. – 1985. – 236 p. 9. Wasser S.P. Mushroom polysaccharides in human health care/ S.P. Wasser, M.Y. Didukh // Biodiversity of fungi. Their role in human life. – 2005. – P. 289–328 pp.

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**Наукове видання**

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