We can see from phylogenetic tree that cluster separation depends neither on geography of virus isolation (Ukrainian isolate are similar to American) nor on plant cultivar as Ukraine isolate was extracted from Sum and substance hosta cultivar whereas similar American isolate was selected from hosta Striptease.

Our data is in agreement with literature data according to with analysis of CP and TGB1 aminoacid sequences of all known HVX strains confirmed their monophyletic origin. Never the less the relationships between different isolates of HVX are still unclear [8].

It was also shown that the HVX-CP gene is less variable than TGB1, which suggests that CP is possibly under more stringent selection pressure than TGB1. The substitutions observed among the isolates in their respective sequences of the two genes were irregularly distributed. The 3'-proximal part of CP was the least variable region. This is probably due to its critical role in mediating essential functions such as interaction with the genomic RNA, movement and encapsidation [9,10].

Observed HVX genetic variability possibly has biological value. There are many instances where it has been shown that a single amino acid change in the CP of a plant virus has a significant impact on virus/host interactions. Hence, additional investigations are required to determine the biological significance of the observed amino acid sequence diversity in CP and TGB1 of HVX.

Reference

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COMPLEMENTARY RESULTS OF LUMINESCENT AND TRANSMISSION ELECTRON MICROSCOPY PROVIDE STRIKING EVIDENCE OF HEAVY METAL IONS' EFFECT ON THE FORMATION OF AGGREGATES OF TOBACCO MOSAIC VIRUS VIRIONS BOTH IN VITRO AND IN VIVO

In vitro electron microscopy studies showed that virus particles treated with heavy metals aggregate creating either "typical" lateral (side-to-side) aggregates of virions or star-like ones not reported previously. Luminescent light microscopy of epidermal cells of virus-infected tobacco plants demonstrated that metal treatment has led to the appearance of mostly amorphous and noncompact inclusion bodies, which were not typical for cells of plants not stressed with a heavy metal. Finally, electron microscopy of thin sections of tissues of virus-infected tobacco plants showed that metal-affected cells contained higher numbers of larger crystalline multilayered inclusions consisting of virus particles.

Key words: virus-infected tobacco, metal-affected cells.

Introduction. Previously we have shown that heavy metal contamination of ecosystems favours plant virus spread [1, 2], more intense accumulation of viruses by systemically infected plants and delay in the onset of virus-specific symptoms [3]. We have also demonstrated positive correlation between the heavy metal content in soil and virus concentration in tissues of plants grown in such soil [4]. Long-term virus passing in heavy metal-stressed plants has been shown to affect neither virus infectivity nor the appearance of local virus-specific symptoms [4, 5]. According to the proposed hypothesis (partially confirmed by the outcomes of laboratory and small-scale field
experiments), chronic effect of abiotic environmental stress factors may lead to intensification of plant virus infection development. Many plausible reasons for this may be suggested including: (i) more efficient intercellular and/or systemic virus transport; (ii) more efficient virus replication/accumulation at the cell level (for instance, due to the plant defenses’ failure); (iii) formation of novel virus variants tolerant to the stress exerted by the metals.

This work has been focused on the second option, as we have studied the in vitro and in vivo effects of heavy metals on Tobacco mosaic virus (TMV) virions and formation of virus-induced inclusion bodies in infected cells.

**Materials and methods.** In this work we have used a well-studied model system “**Tobacco mosaic virus – Nicotiana tabacum cv. Samsun plants**”.

Tobacco plants were virus-inoculated mechanically in two upper leaves at the stage of four true leaves using carborundum powder [6]. The concentration of inoculum was 150 μg/ml. The development of systemic viral infection was monitored visually by symptoms, and using indirect ELISA [7] to measure virus content in the plants (not shown here for the lack of space).

Heavy metals Zn and Pb in the form of water-soluble salts (ZnSO4x7H2O and Pb(NO3)2 (Alfarus, Ukraine) have been used to simulate a soil contamination. The compounds were dissolved in sterile distilled water and added to soil separately (monometal contamination) at the 5X maximum permissible concentrations (MPC). Values of 1X MPC for the metals under study were as follows: Zn – 300 mg/kg, and Pb – 100 mg/kg [8]. The heavy metals were applied to soil 5 days prior to plant inoculation with TMV.

For thin-sectioning studies, leaf tissue was processed, thin sectioned and analyzed with a transmission electron microscope according to generally-accepted protocols [9]. For microscopy, copper grids or blends (Sigma, USA) were coated with chloroform-dissolved 0.2% polyvinyl formaldehyde (Serva, Germany), dried overnight on filter paper at room temperature, and then strengthened with carbon coating. The samples deposited onto grids were stained with 2.5% uranyl acetate and 0.02 N lead citrate (Serva, Germany), and examined using JEM-1200 ex or JEM 1400 (JEOL, Japan) transmission electron microscopes. The sections were photographed at a magnification of 5,000-60,000x.

For luminescent microscopy studies we used fresh leaf tissue of tobacco plants, acridine orange dye and green light filter following generally-accepted protocol [10]. Photographs were made under UV-light at an instrumental magnification of 630x.

For in vitro studies, water-soluble salts of heavy metals (ZnSO4x7H2O and Pb(NO3)2 (Alfarus, Ukraine) have been used at the range of concentrations (5-22 mM, calculated for metal). TMV was used in the distilled water suspension of 0.165 mg/ml. Salt and virus suspensions were mixed 1:1 on a glass slide and incubated at room temperature for 30 min [11]. The resulting suspension was used for transmission electron microscopy studies following the generally-accepted procedure at a magnification of 5,000-30,000x.

**Results and discussion.** In vitro studies showed that TMV particles treated with heavy metals aggregate in two ways creating either ‘typical’ lateral (side-to-side) aggregates of virions known from literature or star-like ones not reported previously (Figure 1, 2). Together with available data [12], the importance of bivalent metal ions for the formation of TMV-specific inclusion bodies in infected plant cells has been suggested.

![Image A](https://via.placeholder.com/150)

(A, B) incubated with Zn salt (instrumental magnification 40000x); (C) incubated with Pb salt (instrumental magnification 30000x); (D) not treated with metal (instrumental magnification 30000x)

Indeed, this has also been confirmed in part by in vivo luminescent analysis of epidermal cells of TMV-infected tobacco plants. Normally, TMV U1 strain induced the development of quasi-crystal intracellular inclusion bodies characterized by compactness, visual homogeneity and geometrically ‘proper’ shape. Zinc treatment has led to the appearance of the inclusion bodies, most of which being amorphous and noncompact, with visible ‘fissures’ (Figure 2).
Further, we were primarily concerned with observing the progress of TMV infection in cells of systemically infected tobacco plants subjected to heavy metal stress. The point was to use electron microscopy as a proxy measure to see whether virus replicates more efficiently in a single cell, and to elucidate the consequences of dual stress at the cell level.

Intact palisade parenchyma cells of tobacco plants had a typical morphology and properly shaped nuclei, chloroplasts and mitochondria. The nucleus with nucleoli normally was located close to the centre of the cell, whereas large chloroplasts were oval in shape, had dense stroma, fully-formed thylacoids and lamellas, and typically resided at the cell periphery. Cells of tobacco plants systemically infected with TMV showed typical mild pathologies mainly involving a moderate vacuolization of the cytoplasm and deformation of chloroplasts. The nuclei of such cells were larger than those of intact cells, but the cell wall, mitochondria and cell membrane did not demonstrate significant alterations. Virus-specific crystalline inclusion bodies have been found in the cytoplasm close to the cell periphery (Figure 3).

Mesophyll cells of Zn-treated TMV-infected tobacco plants demonstrated higher degree of vacuolization. Their organelles (mainly, the nucleus and chloroplasts) were often significantly distorted and usually displaced close to the cell membrane. Virus particles formed numerous prominent crystalline inclusion bodies located close to the cell periphery forming layered structures composed of TMV virions. In Zn-treated cells TMV particles have been repeatedly observed in nuclei and chloroplasts.

Pb-treated plant cells also reacted on viral infection rather similarly (Fig. 7). To our opinion, lead ions had more dramatic effect on the progress of TMV infection in tobacco parenchyma cells. As such, organelles (especially the nucleus and chloroplasts) were more damaged. Cells of virus-infected tobacco plants grown in Pb-enriched substrate also showed higher degree of vacuolization, distinct abnormalities of the cell wall structure (visual 'fragility') and expansion of the intercellular space. These virus-infected cells, as well as Zn-effected, also contained numerous abnormally large starch grains in their chloroplasts. It worth to note that according to microscopy data the cytoplasm of such cells contained more virions and fully-formed virus-specific crystalline inclusions as compared to the cells of Zn-treated virus-infected plants (Figure 4).

In this work we were primarily concerned with observing the progress of TMV infection in cells of systemically infected tobacco plants subjected to heavy metal stress. The point was to use electron microscopy as a proxy measure to see whether virus replicates more efficiently in a single cell, and to elucidate the consequences of dual stress at the cell level.
Visual estimates of the course of viral infection clearly show that heavy metals Zn or Pb potentiate virus accumulation in cells. This is in accordance with the quantitative ELISA results, when Zn and Pb induced respectively 2-times and 4.5-times increase in TMV content as compared to that in virus-infected plants not treated with heavy metals (i.e., representing virus load at the plant level). In addition, the microscopy provided evidence that metal-affected cells contained higher numbers of larger crystal-line multilayered inclusions consisting of TMV particles.

In TMV-infected parenchyma cells of tobacco plants stressed by another stressor, heavy metal (Zn or Pb), similar pathological changes were observed: vacuolization, some distortion/damage of major organelles (nucleus and chloroplasts) more evident in Pb-treated cells, occurrence of large starch grains in the chloroplasts and trans-/malformation of the cell wall (Pb-specific effect). One can say that the overall cell degradation is much more severe when cells are affected by both TMV and a heavy metal.

Conclusions. In this work we have demonstrated plausible coherence of results obtained using different kinds of microscopy for TMV virions in vitro and viral infection in vivo. Presented data show that heavy metals may have direct effects on virions’ aggregation and also can (indirectly) influence the formation of viral inclusions in the cell. Combined effect of viral infection and heavy metals has more serious consequences for cells of tobacco plants and may be indicative of more efficient virus replication in chronically stressed cells.

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References

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We segregated the bacteriophages with long tails, which have different lytic activity, size and consist of proteins from Pulsatilla pratensis plants. These plants were selected in the Kaniv Nature Reserve.

**Key words:** bacteriophages, natural flora, Pulsatilla pratensis.

**Introduction.** Bacteriophages are the most widespread in the environments with high density of active metabolically bacteria [1]. In phytocenosis the reproduction of a set of bacteria which significantly influence a physiological condition of plants is supported and can show phytopathogenic properties. In this regard studying of the interconnected processes of ecology of bacteria and their phages is an actual task. Despite a large number of the works dedicated to segregation and identification of phages, the processes associated with their ecology and evolution in the nature are studied deficiency [2]. Evolutionary approach allows to study them under the influence of environment where the titre of phages in an ecosystem, density of bacterial population and a physiological condition of microorganisms are important factors. Research of interaction of system of populations of phages and bacteria in the conditions of natural environment gives the chance to research the dynamics of interaction and development of populations under the influence of environment factors. It's allows a better understanding about bacteriophages. The relation of quantity of virus particles to quantity of bacteria in averages 10:1. Moreover, the phages are the most numerical organisms on Earth according to some data. Population of phages exceeds $10^{30}$ virus particles [3]. Transduktion is an improbable event [4]. But, with such number of phages, it happens quite often. The number of virus particles consists not only in their huge number, but also in a specific variety [5].

**Material and methods.** Sampling for analyses of features of segregation made from the rhizomes of plants of *P. pratensis* and soil around the roots during the period from June, 2011 to June, 2013. In researches used isolates of bacteriophages which are susceptible to cultures of bacteria of *Pseudomonas fluorescens* and *Seratia marcienses* L-2. After series of passages, pure phage clones with different phage plaque morphology were obtained for every isolate. Viruses accumulated on the cultures in nutrient broth with aeration at 25 °C. In researches used lysates with concentration $10^{9}$ - $10^{10}$ plaque forming units per ml (PFU/ml). Titers were determined by agar-layer technique by Gratia [6]. For research of a range of lytic activity used pathogenic for plants cultures of bacteria: *Erwinia carotovora* 216, *Pseudomonas syringae* pv. atrofaciens 1025, *Pseudomonas viridiflava* 8868, *Pseudomonas fluorescens* 8573 and *Seratia marcienses* L-2, the cultures were provided by the museum of phytopathogenic bacteria of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine. The protein composition of the isolated viruses was researched by electrophoresis by Laemmli [7]. Morphology of virus particles researched by means of electronic microscope (JEOL JEM – 1400).

**Results and discussion.** Phages from samples formed small clear negative colonies on *Seratia marcienses* L-2 and colonies with an aureole on *Pseudomonas fluorescens* with diameter about 0,5-1 mm (fig.1). Research of features of segregation of phages from rhizomes of plants and soil around the roots showed existence of variety of isolates of phages on the basis of lytic ability to used indicator bacteria. The received isolates were characterized by high titers of lytic activity. In total, 4 phage isolates were isolated and described. For determine of a range of hosts was conducted the research of lytic activity of phages on 5 strains of phytopathogenic bacteria. It is revealed that from four checked samples two (the sample №1 and №6) showed lytic activity to strains of different genus of phytopathogenic bacteria (*Pseudomonas* and *Seratia marcienses* L-2) while other two isolates was monovalent (samples №2, №3) – showed lytic activity only to one bacterial strain (tabl.1).

![Fig.1. Negative colonies of phages on the culture: Seratia marcienses L-2 – sample №1 (A), sample №2 (B), sample №3 (C), sample №6 (D) and Pseudomonas fluorescens – sample №1 (E) and sample №6 (F)](image-url)