PALACKY UNIVERSITY IN OLOMOUC

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SPREADING OF POTYVIRUS INFECTION IN PLANT

BACCHALOR THESIS

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Olomouc 2011 **Supervisor: Prof. RNDr. Milan Navrátil, CSc.**

I declare that I wrote this thesis myself with the supervision of Prof. RNDr. Milan Navrátil CSc.

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Abstrakt

Zucchiny yellow mosaic virus (virus žluté mozaiky cukety) je každoročně zodpovědný za významné celosvětové ztráty v produkci tykvovité zeleniny. Hlubší studium molekulárních interakcí a mechanizmů vedoucích k šíření a distribuci viru v rostlinách může přispět k lepšímu porozumění vývoje infekce a také její následné kontrole. Tato bakalářská práce si dává za cíl pozorovat šíření viru žluté mozaiky cukety v tykvi obecné (*Cucurbita pepo* L.) a poznat změny v koncentraci viru v různých částech rostliny během vývoje systémové infekce.

Abstract

Zucchiny yellow mosaic virus is responsible for significant loses in yield and quality of cucurbitaceous vegetable worldwide. The further research of molecular interaction and mechanisms that leads to virus movement and its distribution inside the plant can contribute to better understanding of infection development and subsequent disease control. The thesis aims to observe the spreading of zucchini yellow mosaic virus in Zucchini squash (*Cucurbita pepo* L.) and to illuminate virus concentration changes in different parts of the plants during the systemic infection.

CONTENTS

LIST OF ABREVATIONS

6K1	6kDa protein 1
6K2	6kDa protein 2
AC	asymmetric chlorosis
bp	base pairs
c	concentration
CI	cylindrical inclusion protein
CMV	*Cucumber mosaic Virus*
CP	coat protein
Cys	cysteine
DAS-ELISA	Double Antibody Sandwich Enzyme Linked ImmunoSorbent Assay
DEPC	diethyl pyrocarbonate
DPI	days post inoculation
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	eukaryotic translation initiation factor 4G
F	first grown leaf
G	leaves inoculated by gun bombardment
GFP	green fluorescent protein
HC Pro	helper component protease
IC	inoculated cotyledon leaves
ICTV	International Committee for Taxonomy of Viruses
ICTVdB	International Committee for Taxonomy of Viruses database
kb	kilobase
LC	leaf crumple
M	mechanically inoculated leaves
MC	marginal chlorosis
mN	marginal necrosis
MP	movement protein
NIa	nuclear inclusion protein A
NIb	nuclear inclusion protein B
nt	nucleotide

P1	P1 protein
P3	P3 protein
PAC	prominent asymmetric chlorosis
PD	plasmodesmata
pN	prominent necrosis
qRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
RT PCR	real time polymerase chain reaction
RTM	RTM genes
sN	small necrosis
TEV	*Tobacco etch virus*
TMV	*Tobacco mosaic virus*
UC	uninoculated cotyledon leaves
Y	yellowing of leaf
ZYMV	*Zucchini yellow mosaic virus*
TuMV	*Turnip yellow mosaic virus*

INTRODUCTION

This thesis should illuminate the mechanisms of spreading and the symptom development of *Zucchini yellow mosaic virus* (ZYMV) in Zucchini squash *Cucurbita pepo* L. (Fig. 1). Zucchini squash is one of the host plants of ZYMV in Czech Republic. ZYMV is one of the most dangerous viruses of plant the family *Cucurbitaceae*. The virus affects mainly plants of the class *Cucurbitaceae*. Infection decreases the production which leads to significant economic losses. The virus can be found mainly in south Moravia but during last decade it was spreading from Moravia to Silesia and last years even to some parts of Bohemia. Globally the virus is very variable and occurs all around the world. The virus was firstly characterized in northern part of Italy by Lisa *et al.* (1981).

Molecular aspects of ZYMV local and systemic movement are still the objects of scientific researches and need more attention. Therefore some review chapters summarize the molecular mechanisms and features discovered among the genus *Potyvirus*.

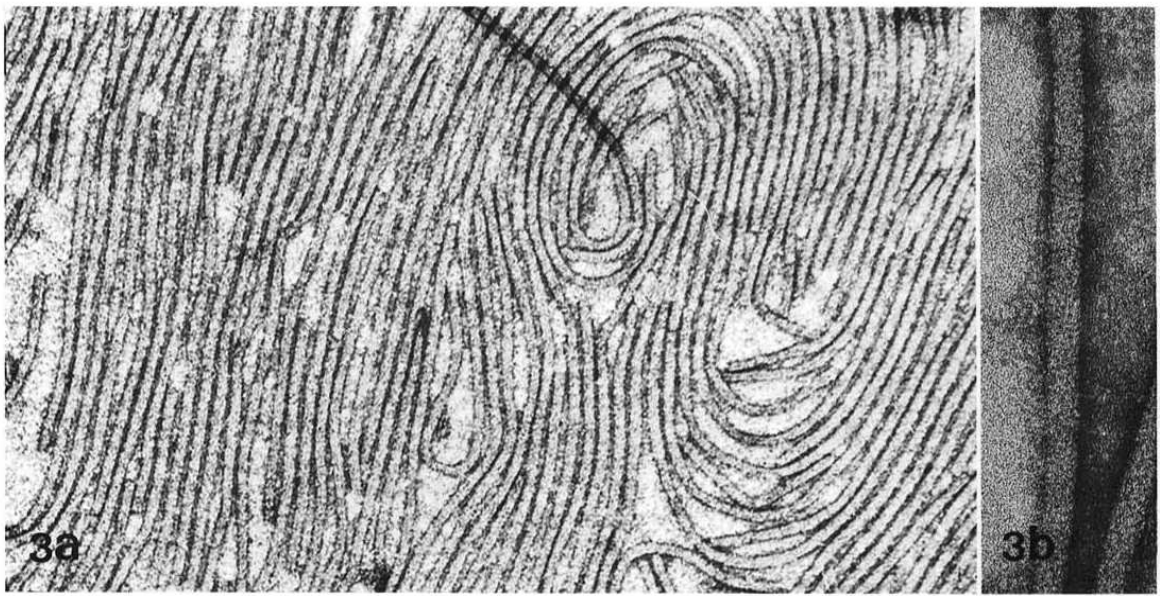


Fig. 1: Electron micrograph of purified ZYMV a) 110 000x b) 370 000x (Lisa *et al.*, 1981).

1. CHARACTERISTICS OF ZYMV

1.1. Classification

The present classification system designed by International Committee for Taxonomy of Viruses is used since 2009. ZYMV can be classified as member of family *Potyviridae,* genus *Potyvirus,* species *Zucchini yellow mosaic virus* (International Committee for Taxonomy of Viruses, 2009). The order is still unassigned. Virus code in ICTVdB is 00. 057. 0. 01. 077. NCBI taxon identification number is 12232.

1.2. Morphological properties of ZYMV

Virions are encapsidated possessing helical symmetry. The capsid is made from flexuous, filamentous particles with the length of 750nm and 12-15nm in diameter. The virus is non- enveloped. Virions are composed of one structural protein (Lisa *et al*., 1981).

1.3. Host plants of the ZYMV

Natural host of ZYMV belongs usually to family *Cucurbitaceae.* The most important are the zucchini squash *(Cucurbita pepo* L.), watermelon (*Citrullus lanatus* Thunb.), muskmelon (*Cucumis melo* L.), cucumber *(Cucumis sativus* L.). These representatives are able to cause severe systemic diseases (Lisa *et al*., 1981). Later, it has been proved that the some of the susceptible hosts are present in the other families e. g. *Chenopodium amaranticolor* (Coste & Reynaud), *Chenopodium quino*a (Willd.) etc. from *Chenopodiaceae* or *Ranunculus sardous* (Crantz) from *Ranunculaceae* and *Senecio vulgaris* (L.) from *Asteraceae* (International Commitee for Taxonomy of Viruses, 2006).

1.4. ZYMV transmission

The virus is transmitted by the vectors from the family *Aphididae* in a non persistent manner (Lisa *et al*., 1981; Lecoq *et al.*, 1981; Aldrez, 1987). The transmission occurs as a highly specific interaction between stylet and viral proteins (Ammar *et al.*, 1994).

1.5. Symptoms

The common symptoms of the ZYMV infection are local lesions, chlorotic mosaic, vein netting, yellowing and stunting. Necrosis develops in the late stage of the infection (Lisa *et al.*, 1981).

1.6. Genome and its expression

Genomic structure can be categorized like positive sense single stranded RNA with linear structure (Revers *et al.*, 1999). Complete genome sequence is usually about 9600 nt long (Glasa and Pittnerová, 2006).

Genomic sequence is translated to polyprotein precursor of molecular mass assigned to 351 kDa. The length of polyprotein was established 9.6 kb (Revers *et al*., 1999). Thereafter the polyprotein precursor is processed into 10 functional proteins which are cleaved by self-encoded viral proteases into several functional proteins (Fig. 2). The proteins composition is common (homologous) to all potyviruses.

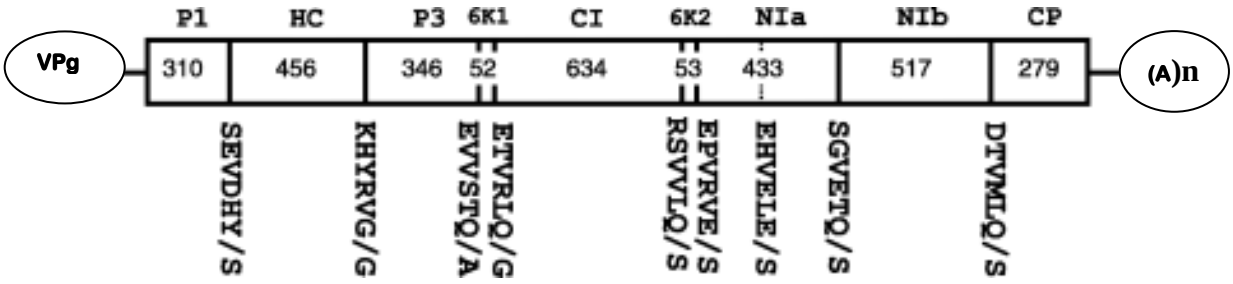


Fig. 2: Polyprotein genome map of the ZYMV-Kuchyna isolate. The amino acid sequences of the putative cleavage sites are shown under the schematic representation of the genome. The length in amino acids of each functional product is indicated in the diagram (Glasa and Pittnerová, 2006).

The **P1 protein** (P1) is the most variable part of potyviral genome objecting to molecular mass and therefore has a potential to be used to distinguish the potyviruses. P1 is autocatallytically active serine type protease that enables the cleavage between cylindrical inclusion protein (CI) and HC Pro (Verchot *et al.*, 1991). P1 proteinase is also known as N-terminal protein. It participates with P3 protein in tolerance braking (Wisler *et al*., 1995).

The **helper component protease** (HC-Pro) is involved in an aphid transmission. The discovery was supported by the evidence that HC Pro serves as a link between the stylet of the aphid and the virus (Ammar *et al.*, 1994). The mutation in the sequence leads to

defects in aphid transmissibility or may result in complete loss of the function (Huet *et al.*, 1994; Canto *et al.*, 1995). The presence of non-specific RNA binding domain supports the hypothesis that HC Pro interacts with RNA and therefore participates in virus replication. Involvement of HC Pro in systemic movement was studied by Cronin in 1995 (Maia *et al*., 1996). The protein was structurally characterized by Plisson *et al.* (2001). The cystein protease activity was assayed by Carrington in 1989 (Lin *et al.*, 2007). The substitution of Cys-Cys-Cys region of HC Pro boxes caused the delay in the cell to cell movement and replication. The systemic spread of the virus was completely abolished. The observations have suggested that the defect was present in sieve elements or in the exit of vascular tissues (Cronin et al., 1995). The evidence points to the association of HC Pro with symptomatology, particularly with severity (Pruss et al., 1997; Desbiez et al., 2010). The protein plays role in post transcriptional gene silencing as a suppressor of genes (Anandalakshmi, et al., 1998; Wu et al., 2010). The suppression of RNA interference molecules leads to significant changes in plant metabolism and therefore in growth and development. The changes usually culminate in symptom development.

The **P3 protein** (P3) has a potential to participate in virus replication and movement because of it's the presence in inclusion bodies and possible interaction with other replication involved proteins (Jenner *et al.*, 2003). Recently, the P3N PIPO protein has been identified as part of P3 as plus two frameshift sequence (Chung *et al*., 2008). It has been proved that P3 is avirulence determinant for TuMV (Jenner *et al.*, 2003). The central Europe isolates (Fig. 4) revealed very low variability between isolates, based on the analysis of P3 protein (Glasa and Pitnerová, 2006). The highest diversity within central Europe isolates was observed in P1 protein. The results are supported by the fact that the other isolates from overseas possess the most significant divergence in the P1 protein sequence.

The **coat protein** (CP) or capsid protein plays major role in encapsidation of viral nucleic acid and in the regulation of viral RNA amplification. But it also participates in transmission by aphids, cell-to-cell and systemic movement. Coat protein is encoded at the C-terminal of polyproteins (Fig. 2). Molecular mass assigned 31 kDa. CP is widely used for phylogenetic analysis (Shukla *et al*., 1991; Rybicki and Shukla, 1992). Coat protein coding region is one of the most variable parts of the potyviral genome but the most of the ZYMV isolates has highly identical amino acid sequence usually over

90%. The exception was found in Reunion and Singapore isolates (Zhao *et al.*, 2003). The amount of CP in the cell correlates to symptom severity (Choi. *et al.*, 2003). The role of CP in cell to cell and systemic movement was searched by Dolja (Maia *et al*., 1996). The CP is three domains protein. N-terminal domain is involved in aphid transmission. Alternation in the conserved amino acid sequence leads to abolishment of the transmission (Atreya *et al.*, 1991). The mutations in some parts of the domain slow down the cell to cell movement (Dolja *et al.*, 1994, 1995). C-terminal forms filamentous capid particularly the core subunit structure (Allison *et al.*, 1986; Domier *et al.*, 1986; Shukla *et al.*, 1988; Dolja *et al.*, 1991). The conserved part of the domain interacts with viral RNA (Shukla and Ward, 1989**;** Dolja *et al.*, 1994, 1995). The CP of ZYMV functions as the determinant of Avr genes (Ullah and Grumet, 2002).

The **cylindrical inclusion protein** (CI) contributes to virus movement and replication. RNA nucleotide binding motif suggests a helicase activity (Fernandez *et al.*, 1995, 1997; Gomez de Cedron *et al.*, 2006). On the beginning of the infection the cone shaped inclusions are observable anchored in the cell wall or on the membrane close to plasmodesmata (Langenberg, 1986; Lawson and Hearon, 1971; Rodriguez-Cerezo *et al.*, 1997; Roberts *et al.*, 1998). As the infection progress the pinwheel-shaped structures aggregate in the cytoplasm (Lesemann, 1988).

The **nuclear inclusion protein A** (NIa) a virus genome linked protein, is 49kDa long. The protein is composed of 2 domains. Proteinase domain is situated on the C-terminal. N-terminal possesses VPg domain (Murphy *et al.*, 1990; Dougherty *et al.*, 1993) The VPg is transported to nucleus. The RNA binding protein plays important role in replication, translation, systemic and local movement. VPg is linked with 5´end of virus RNA. This is a diagnostic feature of picornavirus superfamily (Koonin *et al.* 1993). It is thought that it acts as primer for RNA replicase and that it interacts with RNA polymerase (Fellers *et al.*, 1998). It was proved that VPg acts as virus avirulence determinant.

The **nuclear infusion protein B** (NIb) is RNA directed RNA polymerase involved mainly in maturations of the replication associated protein and capsidation (Darros *et al.*, 1999). Both nuclear inclusion proteins aggregate in nucleus to form crystal shaped bodies, nuclear inclusions.

Other proteins found in the viral genome are **6 kDa protein 1**(6K1), **6 kDa protein 2** (6K2). Proteins are essential for protheolytic separation of P3 from CI and are suggested

to be involved in viral replication. Potyvirus 6 kDa protein was proposed to have a membrane-binding activity (Restrepo-Hartwig and Carrington, 1994). It cleaves autoprotheolytically from VPg. 6kDa protein stays in cytoplasm but VPg is transported to nucleus.

1.7. Phylogenetic relationships

First phylogenetic analysis performed in 2003 suggested the phylogenetic relationships of ZYMV (Fig. 3). The diversity among the ZYMV is associated especially with biogeographical distribution. The analysis was based on sequencing of coat protein. The sequencing distinguished three major groups (Zhao *et al*., 2003). First group involve mainly European isolates, one Californian isolate, isolate form Japan and some Chinese isolates. The leaves display mosaic on the leaves and cause distortion of fruit. Second group abound exclusively in Asia. Observable symptoms on leaves strongly resemble to mentioned first group. Last group include isolates form Reunion, Singapore and other islands from Indian Ocean. The surface of leaves show strong mosaic but the fruit stay symptomless. The most significant difference in genetic diversity was recognized in Asia particularly in China. The cladogram represents results of evolutionary dynamic analysis (Simmons *et al.,* 2008). The scientists, using Bayesian coalescent approach, suggested that the ancestry of ZYMV dates back no more than 800 years. These results impute the dispersal of virus to human activities. The second cladogram represents more detailed information about phylogenetic relationships of ZYMV isolates found in Central Europe additionally highlighting the placement of isolates we used for our experiment (Fig. 4).

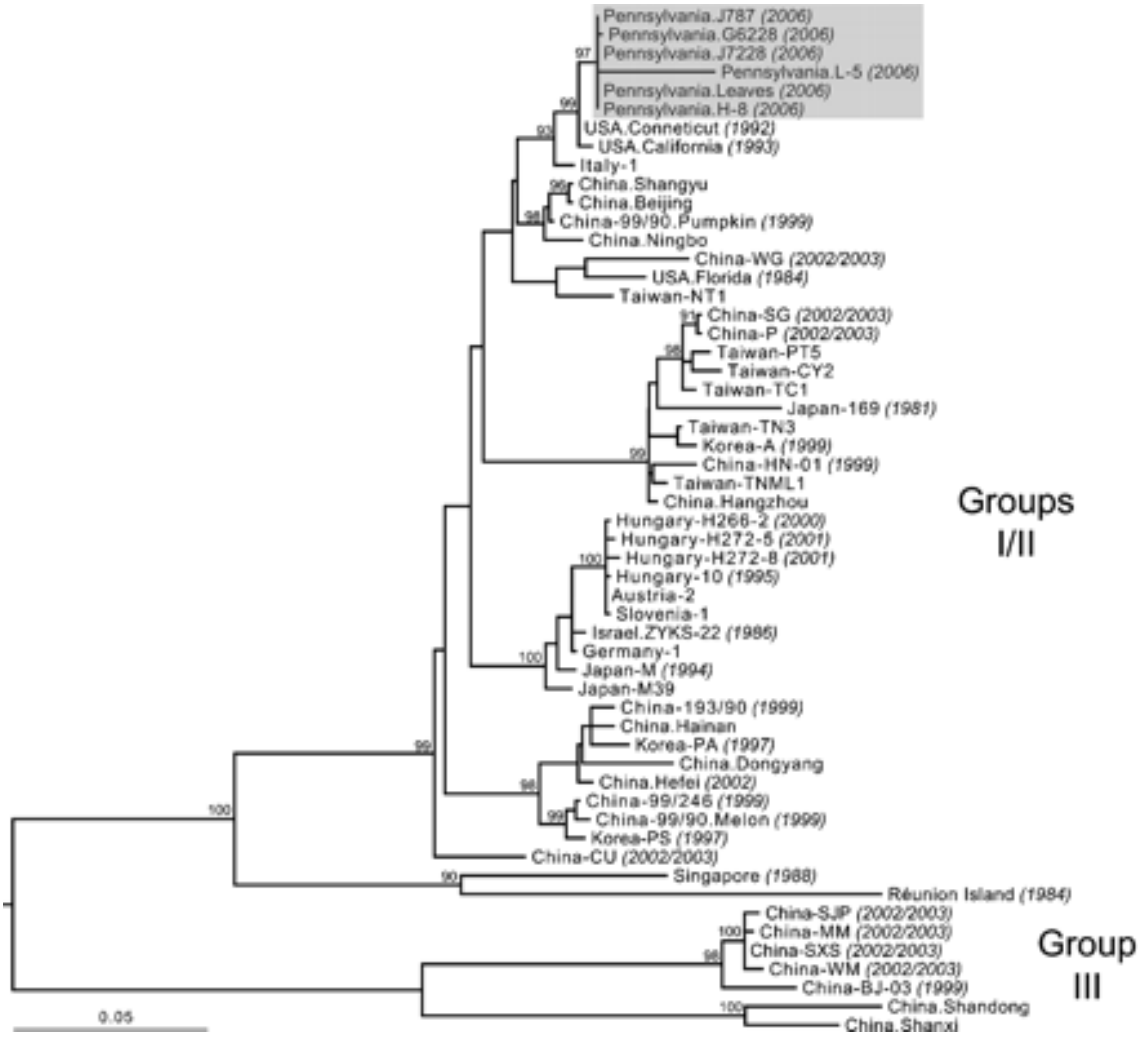


Fig. 3: The tree of 55 ZYMV CP sequences. For viruses where the year of sampling is available, these dates are given in parentheses. Those viruses sampled as part of this study are shaded grey. The group nomenclature depicted represents that previously proposed for ZYMV (Zhao *et al.*, 2003). The tree is drawn to a scale of 0. 05 nt substitutions per site and bootstrap values (>90 %) are shown next to relevant nodes. The tree is mid-point rooted for clarity only.

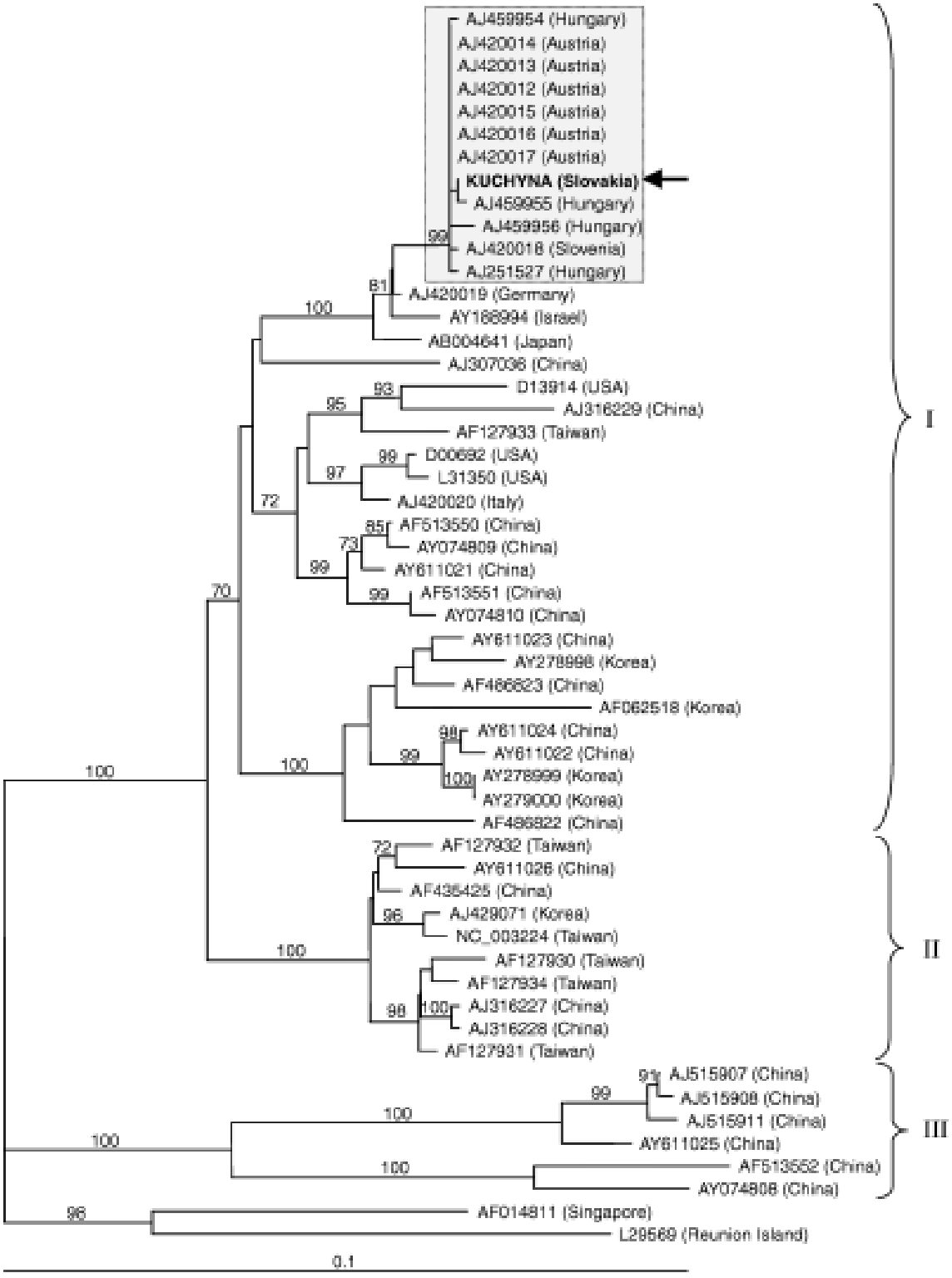


Fig. 4: Phylogenetic tree of ZYMV isolates reconstructed using the complete nucleotide sequence coding for the capsid protein (840 nucleotides). The sequences used were recovered from the GenBank database. The geographical origin of each isolate is mentioned into brackets. Bootstrap analysis with 1000 replicates was performed to

assess the robustness of the branches. Only bootstrap values >70% are shown. The scale bar represents a distance of 0. 1 substitutions per site. The divergent ZYMV isolates from Singapore (AF014811) and Reunion Island (L29569) were used as outgroups. The subcluster of Central European isolates is surrounded by dot and dashed line (Glasa and Pittnerová, 2006). The arrow represents the isolate that is the most closely related to the isolate we were working with.

1.8. Viral replication, gene expression protein processing

ZYMV virions are made of genomic RNA and coat proteins. The precursor is processed into functional products by NIa proteinase, protein responsible for the major cleavage (Li *et al.*, 1997). The P1 and HC Pro cleave autocatalytically (Fig. 5). 6K1 enable separation of P3 from CI then VPg is uridylylated by the polymerase and is covalently attached to the 5'-end of the genomic RNA during post-transcriptional modification. This uridylylated form acts as a nucleotide-peptide primer for the polymerase.

Potyvirus (TEV) replication occurs in the cytoplasm but its replication complexes are associated with endoplasmic reticulum derived membranes (Schaad *et al.*, 1997).

eIF4E is likely to be involved in the onset of viral RNA translation and therefore aids to express all viral proteins and accumulation of virions. It seems to act as a guiding molecule in intercellular transport through plasmodesmata (Lellis *et al.*, 2002; Gao *et al*., 2004).

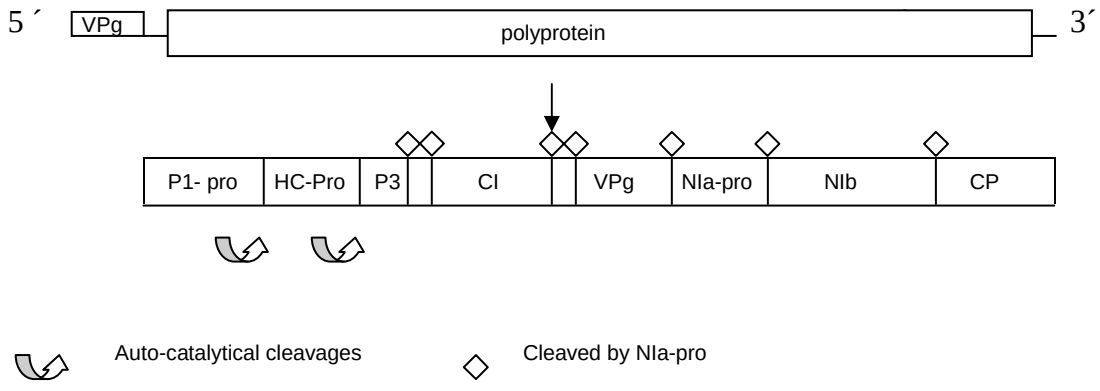


Fig. 5: Polyprotein processing schema of *Potyvirus* with marked cleavage sites of functional products (adapted from ViralZone: www.expasy.org/viralzone, Swiss Institute of Bioinformatics).

2. SPREADING OF POTYVIRUS IN PLANTS

2.1 Cell-to-cell movement

Once the virus penetrates to cell it interacts and begins to spread through the plasmodesmata between the cells. These events require sets of highly specific interactions to overcome natural barriers. Many key mechanisms are still researched and demand further experiments.

Generally the virions or protein complex interacts with docking complex and plasmodesmata. Then size exclusion limit of plasmodesmata is increased due to interplay of specific proteins and inner translocation system is transferred to cytoplasm of adjacent cell to function (Otulak *et al.*, 2010). The groups of movement proteins usually enable the cell-to-cell movement. No specific movement proteins are encoded in potyvirus genome but the cell-to-cell movement requires CP and some additional proteins that play essential role of movement proteins (Scholthof, 2004). The known proteins involved in potyviral cell to cell movement are CI, CP, HC Pro and NIb particularly VPg domain and part of P3, P3N-PIPO (Rojas *et al.*, 1997; Nicolas *et al.*, 1997; Carrington *et al.*,1998; Dunoyer *et al.*, 2004; Wen *et al.*, 2010).

The results of the experiments support the idea that virion formation is necessary for the movement (Dolja *et al.*, 1994, 1995; Roberts *et al.*, 1998). More detailed studies performed on CP significance showed that core domain interacts with viral RNA. Additionally, alternations of the sequence at N-terminal domain slow down intercellular transport (Dolja *et al.*, 1994, 1995). Mutations in central region of HC Pro limited the cell-to-cell movement or in some cases abolished it (Kassachau *et al.*, 1997; Klein *et al.*, 1994). Interaction between HC Pro and CP evoked the rise of size exclusion limit and therefore promoted transport in plasmodesmata (Otulak *et al.*, 2010). Direct evidence for association of CI with plasmodesmata was showed when the transport nucleoprotein complexes inside plasmodesmata contained CP, viral RNA and CI (Rodriguez-Carezo *et al.*, 1997). Further studies lead to the proposal of potyvirus cell-to-cell movement model. The model represents genome interaction with CP to form virion or ribonucleoprotein complex that is directed via CI protein channel to plasmodesmata and then to adjacent cell. The complex is likely to be composed of the CP, CI, NIa, Nib and 6kDa (Fig. 6) (Otulak *et al.*, 2010).

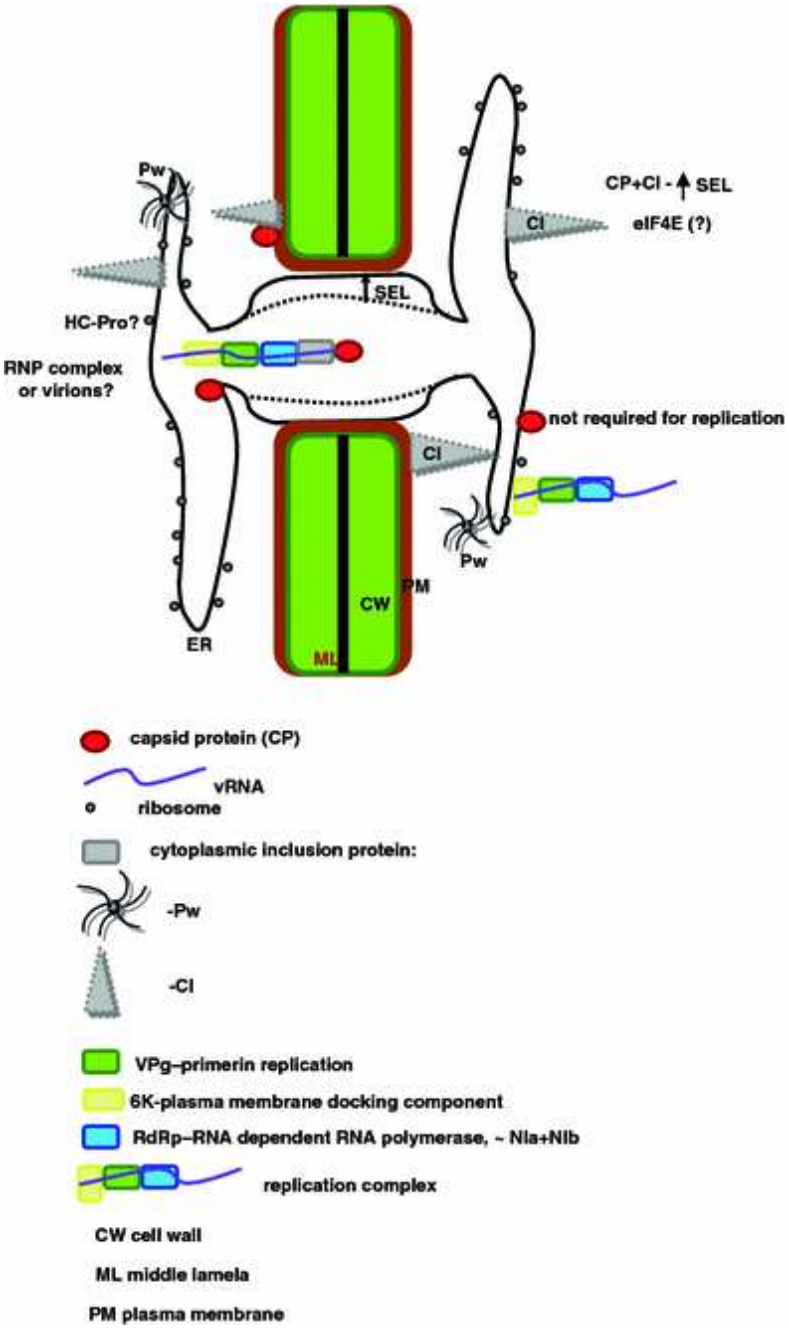


Fig. 6: Potyvirus local transport. Protein complex or virions are translocated to adjacent cell by series of highly specific interactions, which are still under investigation (Otulak *et al.*, 2010). CI - cytoplasmic inclusion, Pw – pinwheel structures, vRNA - viral RNA, SEL - size exclusion limit, RNP complex – ribonucleic acid protein complex.

New model of cell-to-cell movement was proposed when the role of P3N-PIPO protein as movement protein was suggested (Wen *et al.*, 2010). Primary the newborn virions interact with CI at membrane bound sites of replication to form movement complex.

The complex is transported to the aperture of plasmodesma where it interacts with P3N-PIPO protein. The act facilitates the transport of the virion to adjacent cell and leads to formation of conical structures (inclusion bodies) that are typical for potyviruses (Wei *et al.*, 2010).

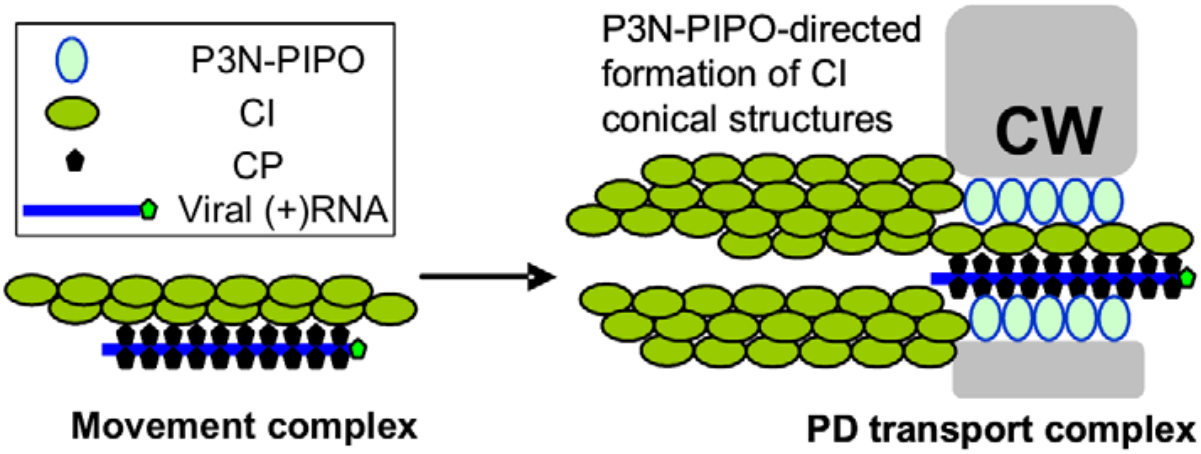


Fig. 7: Model for potyvirus intercellular transport through PD. The virion-CI movement complex is intracellularly transported to the modified plasmodesmata where CI forms conical structures anchored by the plasmodesmata-located P3N-PIPO. The virion is then fed through the CI structures and plasmodesmata to be transferred the adjacent cell, cell wall (Wei *et al.*, 2010). CP coat protein, P3N – PIPO – protein, CI – cytoplasmic inclusion protein, PD plasmodesmata. CW – cell wall.

Eukaryotic translation initiation factor, eIF4E, was found to be susceptibility factor of plant to potyvirus infection. The eIF4E is likely to be involved in the onset of viral RNA translation and therefore aids to express all viral proteins and also to accumulation of the virions. It also seemed to act as a guiding molecule in intercellular transport through plasmodesmata, probably in cooperation with eIF4G (Gao *et al.*, 2004). eIF4E – particle complex promises the onset of the translation processes in adjacent cell due to its association with translation factors via mechanism co-translational disassembly (Gao *et al.*, 2004).

2.2 Systemic movement

The systemic movement of the viruses generally can be characterized as the movement between organs and vascular tissue. The virion or the ribonucleic complex is transported primary by local movement and then through vascular tissue enhanced by phloem loading and unloading along with assimilates and other phloem proteins. The virus must

get inside the sieve element-companion cell complex through parenchyma cells, here it overcomes the specificity.

Plant barriers for tobacco etch virus (potyvirus) infection appeared to be on the interface between companion and sieve cells (Schaad *et al.*, 1996). It was shown that the virus is disassembled before it was transferred to adjacent cell. The ribonucleic transport complex is made. The virions are reassembled in the sieve element (Blackman *et al.*, 1998). The studies based on tracing of GFP suggest that phloem loading is performed in minor veins of the leaves contrary the loading of assimilates was observed only in major veins. The Aphid transmissible viruses are capable of replication in companion cell. The virions that unload of the vascular tissue posse's different composition compared to ones that enters.

Once the complex enters the sieve elements it flows with assimilates to sinks. It has been demonstrated that viral RNAs are capable of interaction with lectins in the stream. The process usually leads to systemic infection of the host that is the result of the plant-virus factor compatible interaction. HC Pro is one of the proteins involved in successful systemic movement of the particle (Cronin *et al.*, 1995; Kasschau *et al.*, 2001). The local movement and replication can be slowed down by the substitution of Cys-Cys-Cys region of HC Pro boxes then the systemic spread of the virus was abolished. It is thought that the defect was present in sieve elements or in the exit of vascular tissues (Cronin *et al.*, 1995). The virus unloading from the phloem occur mainly throught the veins class III. , it has been proved that minor veins function mainly as the xylem transport (Roberts *et al.*, 1997). The RTM genes are responsible for the long distance movement. Any mutation in the sequence of RTM1, RTM2 or RTM3 leads to the dysfunction of the movement of potyviruses (Whitham *et al.*, 1999). The RTM1 and RTM2 genes are expressed in the in the phloem associated tissues. The protein products are found in the sieve elements (Chisholm *et al.*, 2001). The systemic movement is more tightly connected to host factor than cell-to-cell movement. The phloem loading and unloading is therefore influenced by the specific host factors. Synergism in pathology is an event where one strain of the virus co-infects the plant and therefore enables the virus to replicate or moves systemically. This can be represented on the example where the CMV-M was not able to cause the systemic infection because of the plant defence mechanisms that slow down action of CP. But co-infection with ZYMV resulted in systemic infection. This could happen due to suppress of host transcription factors or probably the virus could have used HC Pro of ZYMV to move systemically (Choi *et al.*, 2002).

3. METHODOLOGY

Targeting of ZYMV coat protein (CP) by DAS-ELISA proved the presence of ZYMV in inoculated plants. Quantitative RT-PCR (qRT-PCR) was performed to determine viral concentration in the leaves during infection development.

Plant material and cultivation: Two different species of squash the pumpkin GOLIAS (*Cucurbita maxima* L. cv. GOLIAS*)* and the zucchini (*Cucurbita pepo* L. cv. STARGREEN) were planted. The plants were cultivated on Klasmann 4 substrate. The seeds were obtained from the company Semo a.s., Smržice. Plants grew in the phytotron at 22 °C day / 18 °C night and photoperiod 16/8 hours. It took 8 days before grown cotyledon leaves of pumpkin and over 10 days of grown zucchini's.

***Virus isolates used*:**

ZYMV KUCHYNA - The isolate was obtained from VÚ SAV Bratislava.

ZYMV SRS 7431/97 - The isolate was obtained from State Phytosanitary Administration.

ZYMV H - The isolate was obtained from VÚ SAV Bratislava.

ZYMV TURKEY – The Isolate is part of field collection from Turkey-Antakya-Hatay accomplished by Mgr. Dana Šafářová, Ph.D. The source plants were *C. pepo* (L.).

3.1. Inoculation

Plant viruses were forced to develop strategies to overcome the natural barriers on the surface of the cell, the cuticle and plasma membrane, which protect plants against the pathogens. Naturally the penetration is enabled through injuries usually caused by insect like Aphids. We exceeded these barriers by using 2 different methods of virus transmission. The standard age of the plants for inoculation was 8 days. The *C. pepo* (L.) infected by isolate ZYMV SRS 7431/97 (14 DPI) were used as the source of inoculum.

3.1.1 Mechanical inoculation

One gram of the infected plant tissue was homogenized in 2 ml of phosphate buffer (0. 01 M, pH = 8). The abrasive celit was added to disrupt the tissues on the surface of the leaf. Activated carbon was added to absorb defence organic compounds on the surface. Generally 10 mg of celit and activated carbon was used per 1ml of homogenate. The tissue was disrupted by the sponge soaked in the mixture then the plant was left for 3 minutes and washed.

3.1.2 **Biolistic inoculation (Gun bombardment)**

The method was suggested by Predajňa *et al*. (2010). One gram of the infected plant tissue was homogenized in 2 ml of phosphate buffer (0.01 M, pH = 8). The homogenate was centrifuged 4,500g for 5 min. Supernatant was removed and mixed with carborundum (6-Volume: 1-Weight). 30 µl of inoculum was transfected by airgun to leaf (Fig. 8 and Fig. 9). Plant was left for 3 minutes and washed.

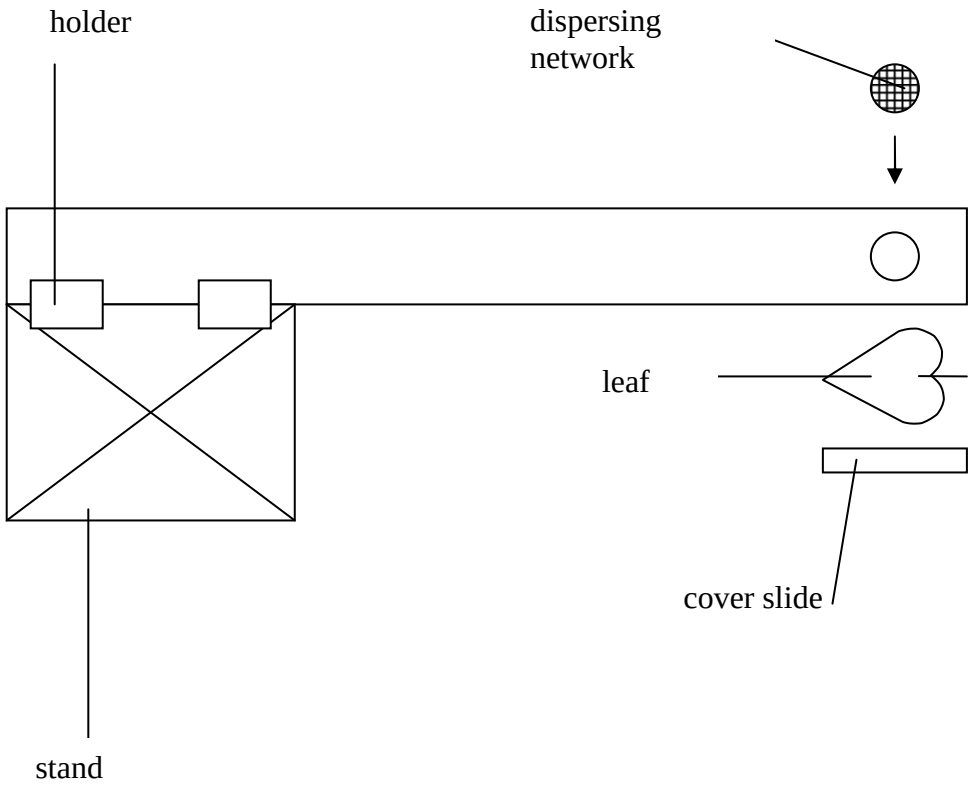


Fig. 8: Schema of plant inoculation using gun bombardment technique.

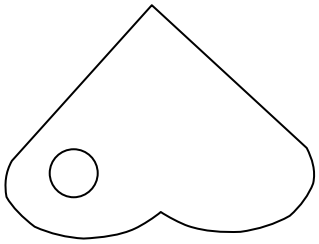


Fig. 9: Sites of inoculum bombardment on the leaf.

3.2. Sample collection, preparation and analysis

The samples were taken regularly from inoculated cotyledon leaf, uninoculated cotyledon leaf and first grown leaf of the plants. Circular samples were cut out by cork knife, which measured 1 cm in diameter. The circles were packed, marked and then put into ice for cooling. Afterwards the set of sample was put into freezer (-80°C). Developments of visible symptoms were regularly noted into table.

Initial sample collection was performed 2 days post inoculation (DPI) from uninoculated cotyledons leaves. The first samples excised from inoculated leaves were collected 4 DPI to assure that virus could replicate and move freely. Primary collection of circles cut from first grown leaves has been done 7 DPI when the leaves grew to reasonable size. The sample of uninoculated plant was collected and used as negative control.

Deep frozen samples were moved from freezer (-80°C) left to melt gradually then the mixed samples were prepared. 4-5 circles of the same characteristic were chosen and cut to quarters. Three fourths of the sample were homogenised and prepared for DAS-ELISA, diluted 1:7. The rest of sample was homogenised and total RNA was isolated. Mass of each sample was noted for subsequent analysis.

3.3. DAS-ELISA

We proved the spreading of the virus in plant by detecting presence of virus at different parts of the plants performing DAS-ELISA (Double Antibody Sandwich Enzyme Linked ImmunoSorbent Assay). The antibodies were produced by LOEWE Biochemica GmbH. The primary antibodies were diluted 1:200 in coating buffer (18µl of IgG solution / 3.6 ml of coating buffer). The 200 µl of diluted antibodies were pipette to microtiter wells. The microtiter plate was incubated at 35 °C for 3 hours then washed

three times by washing buffer. Sample was diluted and homogenized in extraction buffer 1:10 (sample : extraction buffer) and 200 µl of mixture were and to ELISA plate incubated at 4 °C over night. The antibody-antigen complex was formed. IgG-alcaline phosphatase conjugate was diluted 1:10 (18 µl of IgG alkaline phospatase / 3.6 ml conjugation buffer) and 200 µl was pipette to selected pits. The plate was incubated at 35 °C for 3 hours and washed 3 times by washing buffer. Substrate solution was prepared; 3.6 ml of substrate buffer / 2.7 mg of p-nitrophenyl phosphate disodium salt (Na_2PNP). 200 µl was pipetted to selected wells and solution was incubated for 1h in dark. The complex was assayed for the presence of viral specific antigen. The absorbance was gained form ELISA reader after 1 h, 2 h and 24 h. The results were read at 405 nm by ELISA reader BioTek. Set of values were considered as positive if the absorbance exceeded 0.05.

3.4. RNA isolation

Total RNA were purified using NucleoSpin® RNA Plant kit (Macherey-Nagel) in accordance with the manufacture's instructions. The 20-50 mg of plant tissue was homogenised with 350 µl Buffer RA1 and 3.5 µl ß-mercaptoethanol (ß-ME) by FastPrep FP120 (ThermoSavant; speed 5 m/s for 20 s). The lysate was filtered through NucleoSpin® Filter, collected in collection tube (2 ml), and centrifuged at 11,000x g for 1 min to reduce viscosity and to clear the lysate. The RNA binding conditions were adjusted by adding 350 µl of 70% ethanol to the homogenized lysate and vortexed. NucleoSpin® RNA Plant Column was placed to collection tube and the lysate loaded to bind the RNA. The column was centrifuged at 8,000x g for 30 s. The silica membrane became desalted when 350 µl MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000x g for 1 min to dry the membrane. DNase reaction mixture was prepared in a sterile microcentrifuge tube and the 10µl reconstituted rDNase was mixed with 90 µl Reaction Buffer for each of the isolations itself. The tube was flicked and the DNA was digested. The 95 µl of DNase reaction mixture was pipetted onto the centre of the silica membrane of the column and incubated at room temperature for 15 min. Adding of 200 µl Buffer RA2 to the NucleoSpin® RNA Plant Column and centrifugation (8,000x g for 30 s) washed the column and inactivated the rDNase. The 600 µl of Buffer RA3 was pippeted to the NucleoSpin® RNA Plant Column and then centrifuged at 8,000x g for 30 s. The flowthrough was discarded and placed back into

the collection tube. Then 250 µl of Buffer RA3 was added to the NucleoSpin® RNA Plant Column and centrifuged at 11,000x g for 2 min to dry the membrane. The column was put into a nuclease free supplied collection tube. The RNA was eluted in 60 µl DEPC treated deionised water (11,000x g for 1 min.).

3.4.1 Measuring of the total RNA concentration

Spectrophotometer (UV-VIS, Beckman coulter, DU 730) was set to determine RNA concentration and purity of isolated RNA at wavelength 260 nm / 280 nm. The calibration was performed with 2 ml of distilled water before each measurement. Each sample was defrosted and mixed, optionally vortexed and centrifuged, to speed up the defrosting. 5 µl of isolated RNA was added into 2 ml of distilled water.

3.5. RT-PCR

All samples chosen for analyses were DAS-ELISA positive and their absorbance was higher than 1.9 and concentration of total RNA was higher than 0.15 µg/ ml. We verified the presence of viral cDNA at different parts of the plants by performing RT-PCR by detecting viral coat protein (CP) particularly Cter(NIb)-(Nter)CP. Primers were designed by Glasa *et al*. (2007) made by KRD molecular technologies, s.r.o. The reverse transcription of total RNA was performed by AMV Reverse Transcriptase (Promega) using ZY8841R primer of the sequence 5´-TGCTGATGAGACGCTCGTGTG-3´ with negative orientation. Total volume of the reaction for reverse transcription was 40 µl. The reaction mixtures were prepared in flow box. Primary the mixtures were prepared from 9.2 µl of DEPC treated deionised water, 0.8 µl of 20 pmol/ µl reverse primer and 5 µl of sample per one test. Mineral oil added to prevent evaporation. Initial denaturation was performed at 70 °C for 5 min and the cubes were chilled on ice. Second mixture was composed of 12.5 µl of DEPC treated deionised water, 8 µl of RT buffer 5x (M515A, Promega), 2.5 µl of 10 mM dNTPs (C114G, Promega) 1 µl of RNAsin (N251A, Promega) and 1 µl AMV (M510F, Promega) afterwards the cubes were vortexed and centrifuged. 25 µl of second mixture was added to cooled mixture and then incubated at 42 °C for 60min in Thermal Cycler Techne Genet. cDNA was stored in the freezer at -20 °C for subsequent use.

PCR was performed using ZY8841R primer of the sequence 5´-TGCTGATGAGACGCTCGTGTG-3´ with negative orientation and ZY8282F of the sequence 5´- ACA GAG GTC ATT TGC GCT GCG – 3´ with positive orientation, both

designed by Glasa *et al.* (2007). The reaction mixture for PCR was made from 4µl of buffer 5x (M890A, Promega), 10.3 µl of DEPC treated deionised water, 2µl of 2 mM of each dNTPs (R0241, Fermentas, Inc.), 0.25 µl of 20 pmol/ µl reverse primers and 0.25 µl of 20 pmol/ µl forward primers, 0.2 µl of Go Taq polymerase (M830A, Promega). The 3 µl sample was added. The cycling condition set on Thermal Cycler Techne Genet: initial denaturation at 94 °C for 5 minutes, 35 cycles of 94 °C /1 min, 54 °C/45 s, 72 °C/1 min followed by final extension at 72 °C for 10 minutes.

Electrophoresis was performed to analyze products. 50 ml of 1.5 % Agarose gel (AMRESCO, 0491B70) in TAE buffer was supplemented with 4 µl of Gel Red (Biotinum) and filled the electrophoretic vessel. 30% glycerine in DEPC treated deionised water supplemented with 0.1 % bromphenol blue was used as loading solution (4x concentrated). 2 µl of the 100bp DNA ladder was used as a marker (SM0243, Fermentas, Inc.). The 3 µl of PCR products were analysed by electrophoresis. The voltage applied was 80 V for 45 minutes. The results were read by UV SynGen transiluminator using GeneSnap software.

3.6. qRT-PCR

VersoTM SYBR® Green 1-Step QRT-PCR Fluorescein Kit has been used to determine the viral concentration at different parts of the leaves. Primers were designed by Glasa *et al.* (2007) were used; ZY8841R primer of the sequence 5´-TGCTGATGAGACGCTCGTGTG-3´ with negative orientation and ZY8282F of the sequence 5´- ACA GAG GTC ATT TGC GCT GCG – 3´ with positive orientation. qRT-PCR analysis was performed with the Rotor Gene RG-3000A in 25 µl reaction volume. The mixture contained 2µl of buffer 10x (Y02028, Invitrogen) 0.6 µl of 50 mM $MgCl_2$, 11 µl of DEPC treated deionised water, 2 µl of 2 mM dNTP (R0241, Fermentas, Inc.) 0.2 µl of 20 pmol/µl forward primer and 0.2 µl of 20 pmol/µl reverse primer, SYBR Green 0.8 µl (600548, Stratagene), 0.2 µl of Platinum Taq polymerase (10966-034, Invitrogen), and 3 µl of sample. The cycling condition applied: single polymerase activation 94 °C for 2 minutes, then 40 amplification cycles, denaturation 94 °C/45 s, annealing 54 °C/45 s, extension 72 °C/45 s). The melting temperature of products was analysed under following condition of melting 72-95 °C (hold 45 seconds on the 1st step, hold 5

seconds on next steps). Subsequently the agarose gel electrophoresis was performed in TAE buffer at 80 V for 50 minutes including 100 bp DNA ladder (Fermentas, Inc.) that was used as molecular marker. The results were read by UV SynGen transiluminator using Gene Snap software Rotor-Gene 6.0.23.

4. RESULTS

4.1. Inoculations and symptomatology

The ZYMV infection was successful in all inoculated plants. Mechanically inoculated plants showed symptoms at the same time as the ones inoculated by gun. The symptoms were clearly observable on the first grown leaf 7 days post inoculation (Fig. 10). Primary signs suggesting the presence of the virus were marginal chlorosis. Afterwards asymmetric chlorosis progressively developed on the surface of whole first grown leaf. As the infection continued the leaves crumpled. Final stage was accompanied by yellowing of the leaves and necrosis development. Progress of the symptoms was summarized in the table (Tab. 1). The comparison of results from all analysis performed, was summarized in table (Tab. 2). The symptoms of the cotyledons leaves were not observed.

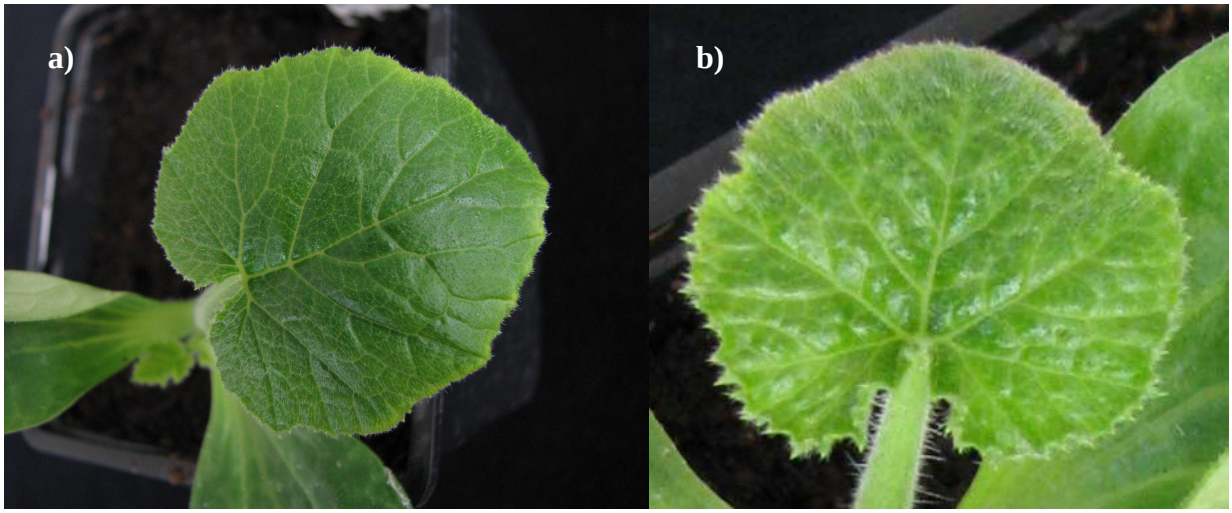


Fig. 10: The symptom development of ZYMV on Zucchini squash (*Cucurbita pepo* L.). a) 7 DPI and b) 11 DPI. Photos by Mgr. Dana Šafářová, Ph.D.

Table 1: Progress of the symptom development on the *Cucurbita pepo* (L.) plants were inoculated by the viral isolate ZYMV SRS 7431/97. DPI – days post inoculation, mC - marginal chlorosis, AC - asymetric chlorosis, PAC - prominent asymetric chlorosis, Y - yellowing of leaf, sN- small necrosis; pN- prominent necrosis, mN-marginal necrosis) LC - leaf crumple

Method of inoculation	**7 DPI**	**9 DPI**	**11 DPI**	**15 DPI**	**42 DPI**
biolistic	MC	AC	MC, AC	AC, LC	soft Y
biolistic	MC	AC	AC	AC, LC	Y
biolistic	MC	AC	AC, sN	AC, LC, sN	Y, sN, mN
biolistic	MC	AC	AC	AC, LC	Y, sN, mN
biolistic	MC	AC	AC, LC, sN	AC, LC, sN	pN
biolistic	MC	AC, LC	AC, LC	AC, LC	strong Y
biolistic	MC	AC, LC	AC, LC	AC, LC	Y, pN
biolistic	MC	AC, LC	AC, LC	AC, LC	Y, mN
biolistic	MC	AC	AC	AC, LC	Y, mN
biolistic	MC	AC	MC, AC, LC	AC, LC	Y, sN
mechanical	AC	AC	AC, LC	AC, LC	Y, mN
mechanical	MC	AC	MC, AC	PAC, LC	Y, sN, mN
mechanical	AC	AC	AC, LC	PAC, LC	Y, sN
mechanical	MC	AC, LC	MC, AC, LC, Y	PAC, LC	Y, sN
mechanical	MC	AC, LC	AC, Y, sN	PAC, LC,sN	Y, sN
mechanical	MC	MC, AC, LC	MC, AC, LC	PAC, LC	Y, sN, mN
mechanical	MC	AC, LC	AC	PAC, LC	Y, sN
mechanical	AC	AC	AC, LC	PAC, LC	Y, mN
mechanical	AC	AC, LC	AC, LC	PAC, LC	Y, sN, mN

Table 2: Spreading of ZYMV infection in inoculated *C. pepo* L. plants and virus concentration development. AC – asymmetric chlorosis, C – control, DPI – days post inoculation, F – firstly grown leaves, G – gun bombardment inoculation, IC – inoculated cotyledons leaves, UC – uninoculated cotyledons leaves, LC – leaf crumpling, M – mechanical inoculation, mC – marginal chlorosis, PAC – prominent asymmetric chlorosis, sN – small necrosis, Y – yellowing; + positive, - negative, [1] – below detectable level, [2] – sample collection error, [3] – error in mixing, [4]– RNA isolation error, [c] – contamination.

DPI	**Leaf**	**Inoculation**	**Symptoms**	**Sample number**	**DAS-ELISA**	**RT-PCR**	**Relative concentration [%]**
2	UC	G	-	26	-[1]	+	0.01
2	UC	M	-	27	-[1]	+	0.05
4	IC	G	-	22	-[2]	-[2]	0.00[2]
4	IC	M	-	23	+	+	17.70
4	UC	G	-	24	-[1]	+	0.03
4	UC	M	-	25	-[1]	+	0.20
7	UC	G	-	14	-[2]	+	64.90
7	UC	M	-	15	-[2]	+	113.83
7	IC	G	-	12	-[1]	+	0.01
7	IC	M	-	13	+	+	11.65
7	F	G	mC	10	+	+	0.72
7	F	M	mC, AC	11	+	+	0.01[3]
9	UC	G	-	4	+	-[2]	5.57
9	IC	G	-	5	+	+	0.87
9	IC	M	-	2	+	+	1.30
9	F	G	AC, LC	3	+	+	6.88
9	F	M	AC, LC	1	+	-[4]	0.00[4]
9	UC	M	-	30	-[1]	+	0.01
9	UC	G	-	29	-[2]	+	7.61
11	F	G	AC, LC, sN	6	+	+	0.01[3]
11	F	M	AC, LC, sN, Y	7	+	+	13.37
11	UC	G	-	9	-[1]	+	0.02
11	IC	M	-	8	+	+	8.43
11	UC	M	-	28	-[1]	+	0.01
15	IC	G	-	19	+	+	78.43
15	UC	G	-	20	+	+	222.48
15	UC	M	-	16	+	+	19.34
15	F	G	AC, LC, sN	21	+	+	80.89
15	F	M	PAC, LC	17	+	+	9.54

DPI	Leaf	Inoculation	Symptoms	Sample numer	DAS-ELISA	RT-PCR	Relative concentration [%]
15	IC	M	-	18	+	+	0.46
-	C-	*Nicotiana glutinosa*	-	31	-	+[c]	0.00
	C+	SRS	AC	32	+	+	100.00
	H_20		-	33		-	0
	H_20		-	34		-	0
	H_20		-	35		-	0
	H_20		-	36		-	0

4.2. ZYMV presence detection

The DAS-ELISA test proved that the virus was present in majority of inoculated cotyledon leaves (Tab. 2). It is likely that an error occurred during collection of sample number 22 (4 DPI). The fact that sample collection error occurred is supported by the results of RT-PCR and qRT-PCR. Low sensitivity of DAS-ELISA test explains the false negative result of inoculated cotyledon leaves (sample number 12) and the absence of virus in uninoculated cotyledons leaves. The positive result of DAS-ELISA was guesstimated to approximately 0.46% and higher relative concentration of virus, obtained by qRT-PCR. Firstly, the presence of virus was detected in uninoculated cotyledon leaves 9 DPI then the values dropped below the threshold. The re-rise of virus accumulation (sample number 29) was observed 11 DPI but it has not been recognised by DAS-ELISA. The virus in UC was redetected 15 DPI. The virus was present in all firstly grown leaves.

4.3. Validation of the primers

Five chosen samples (2, 14, 15, 23 and 32) were analysed to check functioning of the primers (Fig. 11). The viral cDNA was expected to be present in every sample. The suggestion was based on the results of DAS-ELISA assay. The fragment was not present in sample 15 due to wrong pipetting of the sample when it was removed from cycler, only oil was transferred to electrophoretic chamber. The product was present during re-analysis. The PCR product f the expected length was detected and therefore the primers functioned properly.

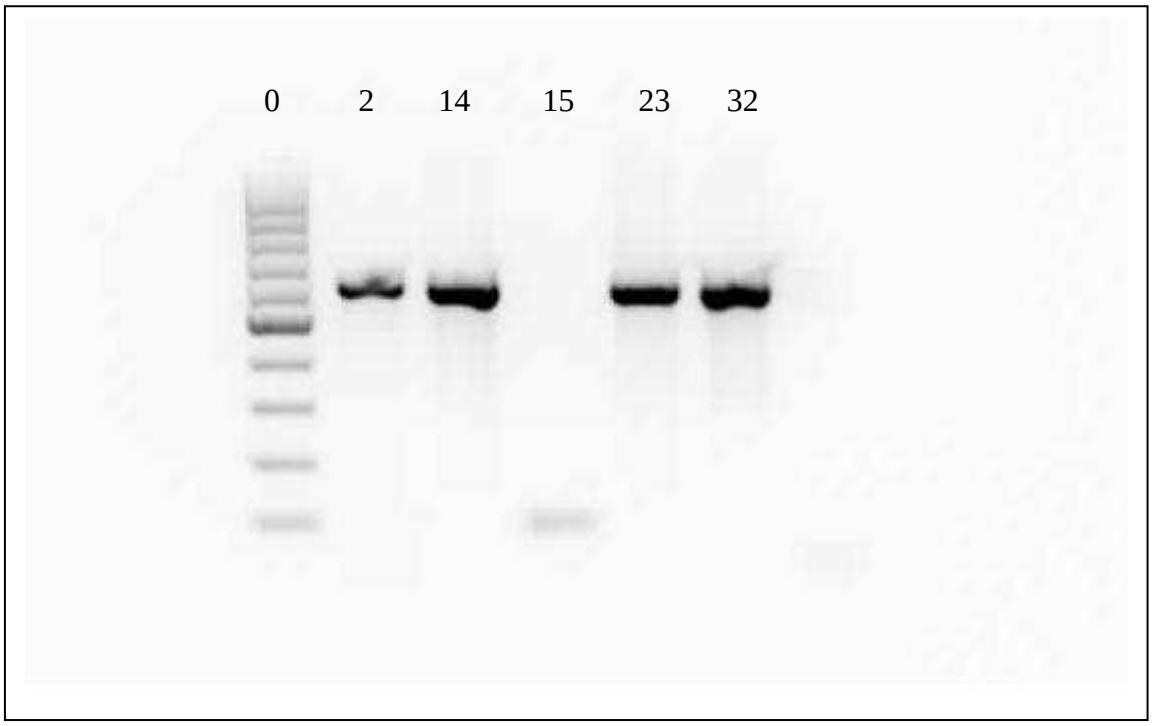


Fig. 11: ZYMV primers validation for qRT – PCR. Five chosen samples were analysed to check the presence of virus. Expected length of PCR product was 559bp.

4.4. Spreading of the ZYMV in *Cucurbita pepo* (L.)

The ZYMV presence was previously detected by DAS-ELISA. The virus was not found in uninoculated cotyledons leaves at early stages of the infection contrary to qRT-PCR results where the virus was detected in low levels, therefore the viral concentration of uninoculated cotyledons leaves was below detectable level of DAS-ELISA.

The analysis of Agarose gel electrophoresis of qRT-PCR showed that the virus moved to uninoculated cotyledons leaves within 2 DPI (Fig. 12) although its accumulation was very low (Tab. 2). The expected length of ZYMV fragment was 559 bp. The ZYMV was found in all samples apart of samples 1and 5 because of pipetting error. The sample 22 was prepared incorrectly for all analysis. The negative control, sample number 32, was contaminated. The other negative controls (sample number 33-36) remained virus free.

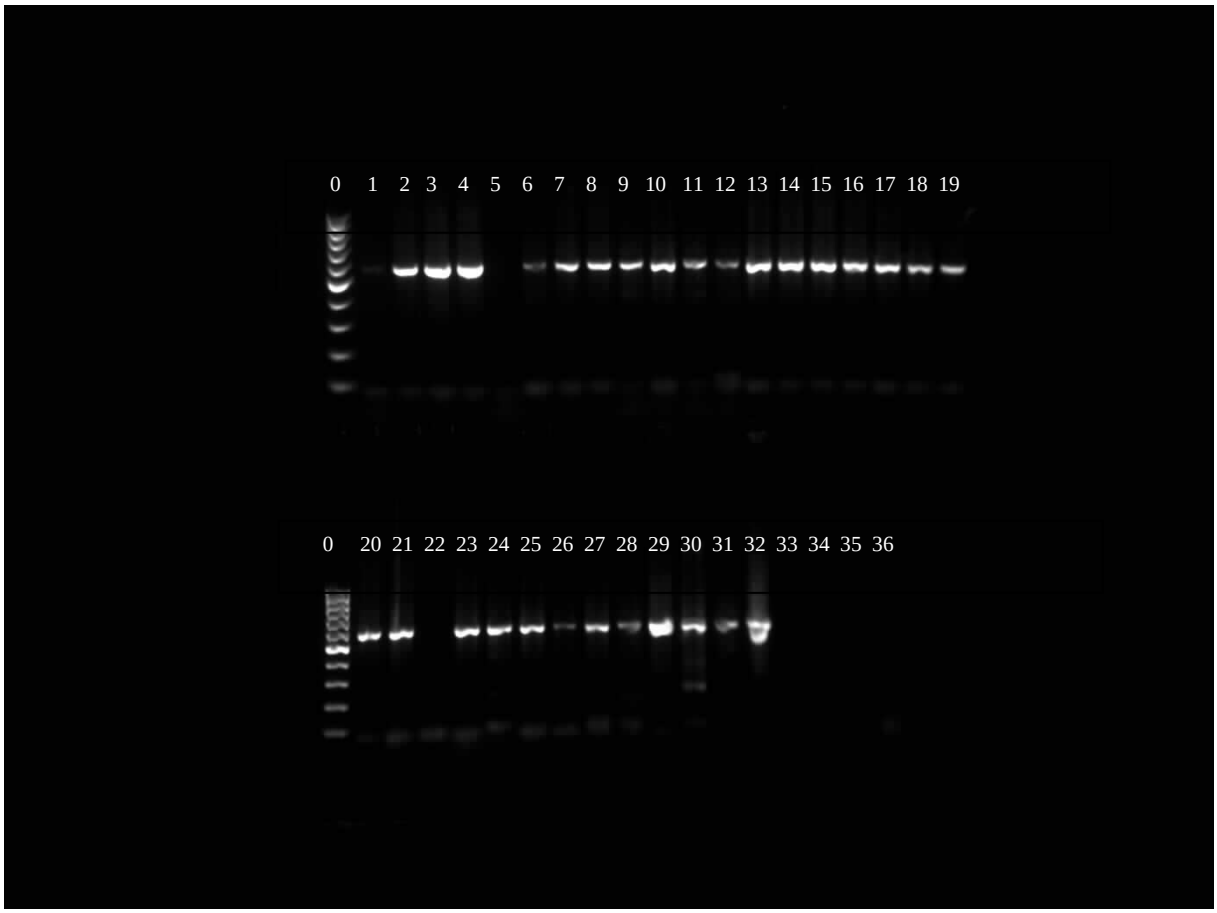


Fig. 12: Agarose gel electrophoresis controlling results of ZYMV qRT-PCR products. Line 0 - molecular ladder (100bp), lines 1-32: sample number 1-32, line 33-36: negative control.

The relative concentration in UCG leaves grew until 7 DPI when the accumulation dropped (Fig. 13). The concentration began to re-increase 9 DPI. The top of 9.54 % was reached 15 DPI. The initial fluctuation was observed in UCM leaves earlier then in UCG leaves. The concentration rose steeply and culminated 9 DPI. Afterwards the decrease to 0.1 % and re-increase up to 0.46 % was noted.

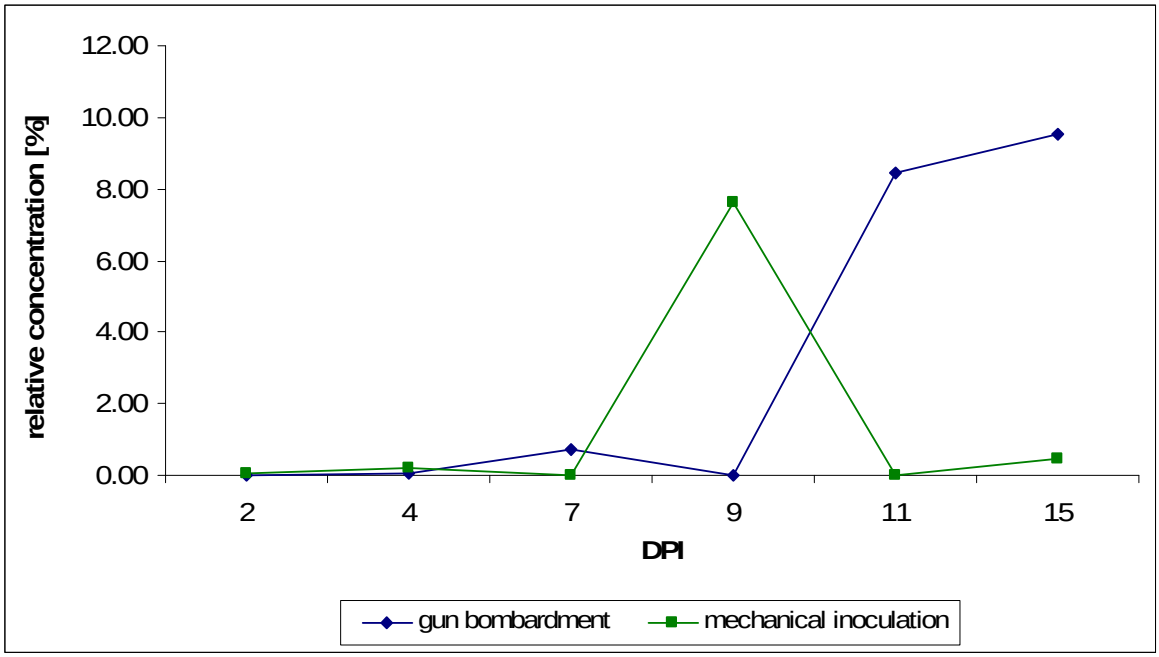


Fig. 13: Relative concentration of ZYMV in uninoculated cotyledons leaves of *Cucurbita pepo* L. plant using biolistic inoculation technique (left) and using mechanical inoculation technique (right). DPI – days post inoculation.

DPI	Sample number	Relative concentration [%]
2	26	0.01
4	24	0.03
7	10	0.72
9	30	0.01
11	8	8.43
15	17	9.54

DPI	Sample number	Relative concentration [%]
2	27	0.05
4	25	0.20
7	11	0.01
9	29	7.61
11	28	0.01
15	18	0.46

Biolistically inoculated cotyledons leaves had quite low initial relative concentrations values, only 0.003 % four days post inoculation. But the concentration was gradually rising and culminated 15 DPI when the relative concentration reached 19.34 %. Unfortunately, the data from 11 DPI are not accessible due to mistake in sample preparation. The mechanically inoculated cotyledons leaves show primary rise of relative concentration up to 17.7 % four days post inoculation then the presence of virus gradually declined (Fig. 14). The re-increase of the virus concentration was noted 15 DPI up to 80.89 % of relative concentration.

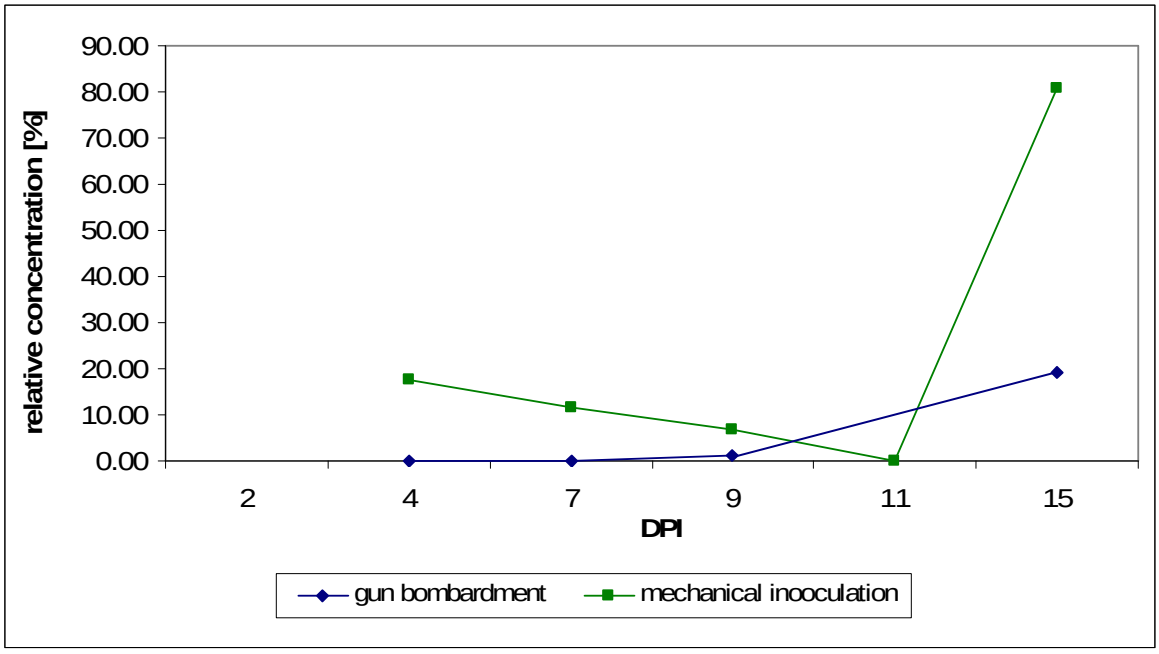


Fig. 14: Relative concentration of ZYMV in inoculated cotyledons leaves in *Cucurbita pepo* L. plants using biolistic inoculation technique (left) and using mechanical inoculation technique (right). DPI – days post inoculation.

DPI	Sample number	Relative concentration [%]
4	22	0.00
7	12	0.01
9	2	1.30
11	Not tested	Not tested
15	16	19.34

DPI	Sample number	Relative concentration [%]
4	23	17.70
7	13	11.65
9	3	6.88
11	9	0.02
15	21	80.89

Concentration of the virus in the first grown leaves was very high 7 DPI, 64 % for gun inoculated plants and 113 % for mechanically inoculated plants (Fig. 15). The sharp fall of both values followed. The biolistically inoculated plants showed lower concentration 11 DPI in comparison with gun bombardment technique of inoculation. The relative concentration of mechanically inoculated plants reached the bottom 9 DPI. The virus recovery was observed 15 DPI where its concentration reached the peaks. The virus accumulation went up to 78 % using gun bombardment technique and reached even 222 % when inoculated mechanically. The movement of the virus was summarized in the Fig. 16.

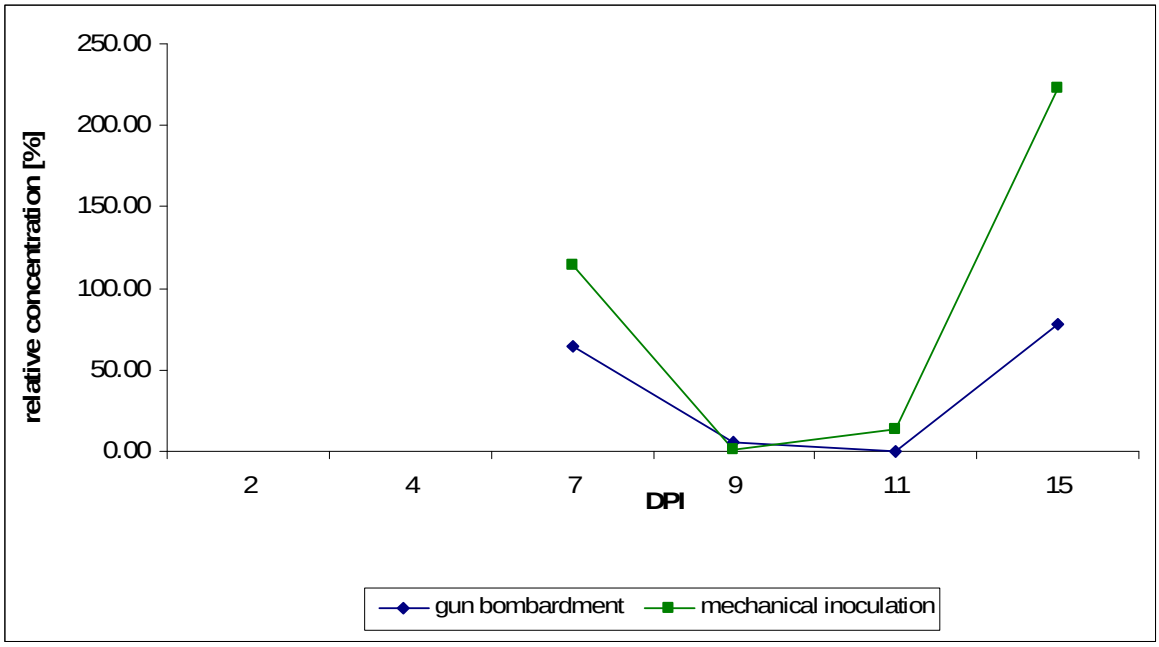
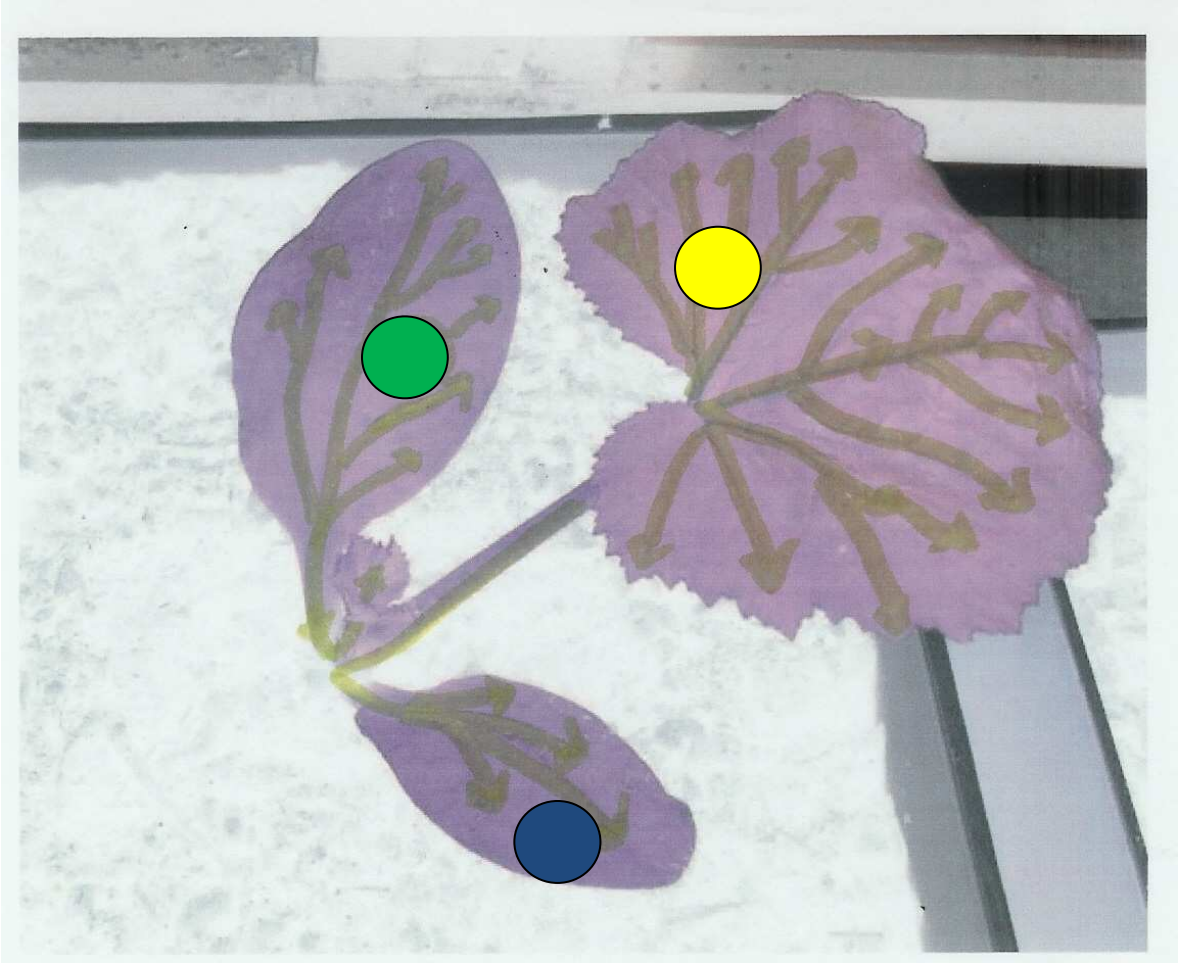


Fig. 15: Concentration of ZYMV in firstly grown leaves using biolistic inoculation technique (left) and using mechanical inoculation technique (right). DPI – days post inoculation.

DPI	Sample number	Relative concentration [%]
7	14	64.90
9	4	5.57
11	6	0.01
15	19	78.43

DPI	Sample number	Relative concentration [%]
7	15	113.83
9	5	0.87
11	7	13.37
15	20	222.48

Fig. 16: The spreading of the virus inside the plant. On the onset of the infection the virus spread among the inoculated cotyledon then it moved to the first grown leaf and to the other cotyledon leaf (uninoculated leaf). The presence of virus was detected by RT-PCR in uninoculated cotyledon leaves (blue circle). The virus was detectable by RT-PCR and DAS-ELISA in inoculated cotyledon leaf 4 DPI (yellow circle). The presence of virus in first grown leaf was primarily assayed 7 DPI. Site of inoculation (green circle).



DISCUSSION

Two inoculation techniques were used to transmit the virus to plants. The gun bombardment technique was more demanded for laboratory equipment than mechanical inoculation technique although it is still very cheap and efficient technique. Major advantage of this technique for our purpose was a minimal disruption of the leaf surface.

Gun inoculation may exceed if the technician is less experienced because the plant shows no observable injuries when the Zucchinis cotyledon leaves were inoculated. The considerable disadvantage of gun inoculation is aerosol contamination of the environment. Generally the concentration of the virus in biolistically inoculated plants was slightly lower than in the others. Mechanical inoculation technique is classical way of inoculation used among the plant scientist, it is cheap and efficient technique but the intense application of inoculum on the leave may cause distinct injuries of the plant tissue and lead to premature necrosis of the tissue. A sufficient surface of the leaves was essential for complete sample collection.

All of the inoculated plants showed symptoms that are typical for ZYMV infection. The marginal chlorosis was found on the first grown leaf surface at early stage of the infection. Subsequently the chlorosis made nettings on the whole leaves surface. The leaves began to crumple and small circular necroses were distinguished. The similar were characterized by Lisa *et al*. (1981). The comparable symptoms development was observed by Wang *et al*. (2004). The yellowing in the late stage of the infection was caused by chlorophyll degradation as the result of the complex plant pathogen interactions studied by Zechmann *et al.* (2003). The cytological changes that zucchini squash undergoes during ZYMV infection were researched by Radwan *et al.* (2007). These inevitable metabolic changes affects crop production therefore indirectly cause economic losses.

The ZYMV was detected by DAS-ELISA and the Agarose gel electrophoresis of qRT-PCR samples. The virus was not found in uninoculated cotyledons leaves at early stages of the infection using DAS-ELISA contrary to Agarose gel electrophoresis results where the virus was detected. Although the DAS-ELISA is less sensitive the test usually produces more consistent data. The results of DAS-ELISA support the schema that virus moved from inoculated cotyledons leaves to first grown leaves and then it appeared in uninoculated cotyledons leaves where it was detected 15 DPI.

The virus probably moved from inoculated leaves to first grown young leaves (sinks) and minority spread to the other older cotyledon. The similar results were obtained by

Dovas *et al.* (2005), who studied fluctuation in concentration of two potyviruses. The qRT-PCR analysis showed that the initial concentration values found in first grown leaves were high. It is important to consider that first sample collection of first grown leaves was performed 7 DPI when the leaves attained sufficient size. Therefore the relative concentration of cotyledon leaves was detected in advance. Earlier data analysis of first grown leaves was impossible.

The relative concentration measurements of uninoculated cotyledons leaves showed initial slight fluctuation that was followed by sharp rise of virus concentration. The timing of the events varied in 2 days for different inoculation techniques (Fig. 13). The rise of virus accumulation in mechanically inoculated plants started earlier. This could be associated with higher concentration of virus in the mechanically inoculated leaves although the data did not prove it. The sudden drop of the concentration could be explained by temporary prevail of the plant defence mechanisms. The development of the concentration of biolistically inoculated plants support the idea that lower concentration of the virus did not switch so strong defence response. The virus overcame the defence easily and the virus concentration rose. It can be only guessed weather the defence response was delayed. Lower initial concentration in gun bombarded leaves can be explained by the transmission of the virus in lower concentration or by cutting the circular sample from temporary virus free part of inoculated leaf. Higher first relative concentration in mechanically inoculated leaves prompt that virus replicated successfully. Gradual decline suggests ZYMV movement to the other parts of the plant that is supported by the high concentration values in first grown leaves 7 DPI and fluctuation in uninoculated cotyledon leaves. The involvement of plant defence mechanisms is possible. The re-increase of ZYMV concentration of inoculated leaves appear to be sign of plant defence mechanisms suppression starting 11 DPI.

The concentration development of first grown leaves signify the high initial concentration values that gradually decrease due to multiple reasons. The rise and sudden fall of concentration were in consensus with results of the experiment performed by Bachand *et al.* (1998). Arguably the plant defence response acted and it is likely that the virus moved to the other young leaves that grow slowly and represent the influential sinks. Predictably the re-rise was linked to defeat of plant defence mechanism.

The apparent low concentration values in whole plant were observed between 7 - 11DPI. Presumably it can be explained as the time when the plant defence mechanisms are fully active and exceed.

CONCLUSION

Transport of the ZYMV within the plant is directed by set of specific interaction between host and pathogen. The process of translocation of viral particle is 2-step process that requires involvement of pathogen proteins and host factors. The essential event for the virus spreading is local movement that occurs from cell to cell via plasmodesmata. The virions or nucleoproteins complex is translocated through plasmodesmata of due to increase of size exclusion limit and aid of the movement proteins that guide the molecule through. Next stage of the successful spread is the penetration to vascular tissue to systemically invade the plant. The complex has to cross natural barriers like bundle sheet to get into the phloem to move then it invades the companion cells and progress through sieve element via veins to sinks.

- Usage of mechanical inoculation technique evoked the distinct disruption of inoculated plant tissue. The gun bombardment technique did not cause such a bold damage of the inoculated leaf surface.
- The sufficiency of the inoculation was comparable; all of the plants were inoculated successfully.
- Initially the relative concentration of virus transmitted by gun was lower. The difference was brought down as the infection progressed.
- The data suggests that virus left inoculated cotyledon leaf and moved to first grown leaf.
- The virus was detected in uninoculated cotyledon although in very low concentration.
- The results showed increasing concentration of the virus in inoculated cotyledons leaves. That supports the replication and the local movement of the ZYMV in mesophyll cell.
- A presence of the virus in uninoculated cotyledons leaves, first grown leaves and symptoms development serves as the evidence for virus systemic movement. It can be assumed that the virus moved via vascular tissue to sinks.
- The significantly higher concentration of virus was measured in the first grown leaves, young leaves, the sites of assimilate consumptions.
- It is likely that the drop in concentration level between 7 and 11 days post inoculation occurred due to temporal overcome of plant defence mechanisms.

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