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Cultivar specific influence of TBSV infection on tomatoes

Abstract: In this study, the effect of *Tomato bushy stunt virus* (TBSV) infection on *Solanum lycopersicum* three cultivars and the model plant *Nicotiana benthamiana* were investigated. According to our studies, the degree of infection by TBSV varies depending on the cultivar of tomatoes. Cultivars *Better boy* and *Pera* are resistant, while *Money maker* cultivar was tolerant to TBSV infection. These results show that the plants might contain different resistance genes that selectively respond to the TBSV virus and indicate the presence of certain defence mechanisms within the species. Moreover, the analysis carried out using the Chlorophyll Fluorescence Imaging System (ChFI) and fluorometer showed that despite the absence of visual infection symptoms in *Money maker* tomato cultivars, there is an increase in the level of secondary metabolites and a slight reduction of the photosynthesis activity.

Keywords: Chlorophyll fluorescence imaging, photosynthesis, *Solanum lycopersicum cv. Money Maker, Tombusvirus, Tomato Bushy Stunt Virus.*

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Introduction. Viruses trigger various plant diseases, causing a significant damage to crop yields and a variety of plants. Essentially, the process of interaction of a pathogen with a host plant depends on how quickly the plant can mobilize protective mechanisms that implies biochemical and structural responses to prevent pathogen development.

Often virus-infected plants survive by overcoming various visible symptoms of infection on leaves (spotting, mosaic, chlorosis, necrosis, etc.). However, an infection inevitably lowers the yield level [1].

Plant viruses, as mandatory biotrophic pathogens, attack a wide range of plant species using the host cell machinery for protein synthesis, genome replication and intercellular systems to support their systemic spreading. Viral infection generally causes symptoms leading to morphological and physiological changes in infected plant hosts, which always takes on low productivity, such as reducing the host's biomass and crop loss.

The most common viral symptom is a leaf chlorosis, which reflects the altered pigmentation and structural changes in chloroplasts. Viral effect on the structure and function of chloroplasts usually leads to the reduced photosynthetic activity. Since the beginning of the twentieth century it became known that a viral infection inhibits the photosynthesis of the host, which is usually associated with viral symptoms [2] Alterations in photosynthesis are a common strategy of viral pathogenesis to facilitate an infection and create an optimal niche [3]. Disruption of the components and functions of chloroplast can cause the formation of symptoms of chlorosis associated with a viral infection. A number of typical changes, followed by chlorotic symptoms, imply the emergence of chloroplast interactions with the virus. These changes include a chlorophyll fluorescence fluctuation and a reduced chlorophyll pigmentation [4], an inhibited photosystem efficiency, an unbalanced accumulation of photoassimilates [5], changes in chloroplast structures and functions [6], a suppressed expression of

nuclear-coded chloroplasts and photosynthesis-related genes (CPRG) and a direct binding of viral components to chloroplast factors [7].

Chloroplasts have always been one of the main targets of plant viruses. For example, a severe chlorosis on systemic leaves infected with *Cucumber Mosaic virus* (CMV) in *Nicotiana tabacum cv. Xanthi nc* is associated with a decrease in the size of chloroplasts containing a few grana. Moreover, another example shows that a leaf mosaic caused by a viral infection may be related to the location of clustered mesophilic cells with differently damaged chloroplasts [8]. The example also shows that the symptom caused by *Potato Virus Y* (PVY) infection is often associated with a decrease in the number and size of the chloroplast in host plants, as well as with an inhibited photosynthesis [9].

Tomato bushy stunt virus (TBSV) is a representative of the genus *Tombusvirus* in the *Tombusviridae* family. It has a fairly limited range of hosts, mainly infecting several dicotyledonous species in individual families and vegetables. However the experimental range of hosts is wide and more than 120 plant species in more than 20 different families are reported to be susceptible, although in most plants the infection often remains localized around the virus inoculated site [10].

TBSV particles encapsulate a single copy of the single-stranded positive sense RNA (ssRNA) consisting of approximately 4,800 nucleotides (nt). Genomic RNA (gRNA) functions as mRNA for translation of two 5'-proximal genes encoding a 33 kDa protein (P33) and a readable 92 kDa product (P92); both are necessary for a replication [11]. The 41 kDa TBSV capsule protein gene (CP) is translated from subgenomic RNA1 (sgRNA1) and is required for a specific host for an effective systemic spreading. Two nested p22 and p19 genes located near the 3'-end of the gRNA are translated from subgenomic RNA2 (sgRNA2). These genes express proteins of 22 kDa (P22) and 19 kDa (Pl9), respectively [12].

Symptoms induced by TBSV are largely dependent on the host genotype; they can range from necrotic and chlorotic lesions, to systemic mild or severe mosaic, or they can result in a fatal necrosis. Original isolates of TBSV from tomato plants caused a spotting, wrinkling and twisting of systemic leaves showing a tissue necrosis in a systemic infection. The yield of tomatoes can be significantly reduced due to a viral infection. Plants can be stunted, and the proliferation of lateral shoots leads to a dense appearance of infected tomato plants, giving a bushy appearance corresponding to the name of the pathogen.

TBSV systemically affects the plants *Nicotiana benthamiana* and *N. clevelandii* and leads to the systemic necrosis, whereas infection of *N. tabacum*, *N. glutinosa* and *N. edwardsonii* results in the formation of local necrotic lesions [13].

Over the past 40 years, it has been reported that *Tombusviruses* cause significant harm to tomatoes in greenhouses and fields in Italy, Argentina, Mexico, Portugal, Tunisia, US and Spain. TBSV also caused epidemics of diseases in aubergines (Solanum melongena L.) in Tunisia and Spain. TBSV was isolated along with other viruses from an infected salad in Czechoslovakia and Turkey.

Fattouh F.A., 2010 reports a loss of chlorophyll content in *TBSV Egh*-infected *Lycopersicon esculentum* and *Cucurbita pepo* and the observation of significant differences in glucose, sucrose and polysaccharides. Symptoms of TBSV infected cabbages are manifested in the form of delayed plant growth and extensive chlorosis, spotting and necrosis of old leaves. Plants infected at an early age died [14].

One of the hosts of the TBSV virus is a tomato plant (*Solanum licopersicum L*). For the first time this virus was discovered by Smith, 1935 in tomatoes [15], and this is where its name originates, although further studies on tomates were less frequent. Currently there is a wide range of tomato cultivars. Therefore the aim of this study is to determine tomato cultivars that are more susceptible to TBSV infection. In the furtherance of this purpose the cultivars *Money maker*, *Pera*, *Better Boy* were selected, since according to virologists, these particular cultivars were one of the most susceptible to *TYLCV* (*Tomato Yellow Leaf Curl Virus*) infection [16]. The choice of such cultivars is also based on the fact that the influence of TBSV on them has not yet been studied.

Materials and metods

Plant and virus materials: Seeds of plants *Nicotiana benthamiana* and *Solanum lycopersicum* were grown in the soil containing biohumus and basic nutrients such as nitrogen (NH4 + NO3) 150 mg/l, phosphorus (P_2O_5) 270 mg/l, potassium (K_2O) 300 mg/l, with a pH of 6.0-6.5 in the

greenhouse. The greenhouse is equipped with white-fluorescent lamps of 230 V, a timer with 16-hour operation, and a temperature regime of 25/20 ° C (day and night) with a humidity of 80%. Plants were watered 3 times a week with 50 ml of water.

Plants of 25-35 days of the same size were selected to provide equal experimental conditions. 20 μ l of an inoculation buffer (10 mM sodium phosphate buffer, pH 6.9) containing virions and carborandum (d= 0,037мм) were applied on the surface of middle leaves of each plant.

Plants inoculation by TBSV RNA transcripts: For plants inoculation in vitro generated transcripts of full length TBSV cDNAs were used [17]. For this, plasmids containing the inserts were linearized at the 3 -end of the viral cDNA sequence by restriction SmaI enzyme digest. Transcripts were synthesized using T7 RNA polymerase, and resulting transcripts were used for inoculation of plants as previously described [18]. Control plants were mock-inoculated by using phosphate buffer without viral RNA. Healthy and infected plants were grown separately in the same conditions.

Western blot analysis: Protein samples extracted from mock-inoculated and TBSV infected plants were separated by 12% SDS - polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Osmonics, Westborough, MA). After transfer the membranes were stained with Ponceau S (Sigma, St. Louis, MO) for verification of protein transfer efficiency. The resulting membrane was incubated with diluted primary antibodies (1:5000) raised against P19 TBSV protein. Alkaline phosphatase conjugated to goat anti-rabbit antiserum (Sigma) was used as a secondary antibody at a dilution of 1:3000, and the immune complexes were visualized by hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as the substrate.

Measurements of photosynthetic activity and chlorophyll fluorescence: Measurement of photosynthetic activity was carried out at various time points in virus-infected and mock-inoculated plants. Local measurement of photosynthetic activity on upper, non-inoculated leaves was made with a portable fluorometer (FluorPen FP 100, Czech Republic) every 3 days after inoculation during 24 days.

Measurements of a chlorophyll fluorescence induction on infected plants were performed using a commercial kinetic imaging fluorometer (FluorCam, Foton System Instruments, Czech Republic) as described by Perez-Bueno M. et.al. (2006). All measurements were conducted on the sixth or seventh leaf stage of developed plants.

Statistical analysis:

Statistical analysis of the average means of the photosynthesis intensity was carried out using the software GraphPad Prism 6.

Results.

Determination of tomato cultivars susceptible to TBSV infection

Five weeks old tomato leaves were mechanically inoculated with TBSV transcripts as described previously and grown in the greenhouse at 25 ° C. Symptoms visually did not appear on all cultivars at 35 dpi (days post inoculation). Neither the inhibition of growth nor the manifestation of symptoms in the form of twists and yellowing of the leaves were observed for all cultivars *Better boy* (Fig.1), *Pera* (Fig. 2) and *Money maker* (Fig. 3) [19]. TBSV inoculated *N. benthamiana* plants show pronounced symptoms such as leaf curling, yellowing, stunting and the acquisition of a bushy form of stems. For this reason *N. benthamiana* was used as a positive control model plant on which the fundamental mechanisms of RNA interference suppression by P19 protein of TBSV have been previously described [20,21] (Fig. 4).

Five weeks old *N. Benthamiana* plants were rub-inoculated. On the left is the mock-inoculated (by phosphate buffer of virus-free material) plant. On the right is the TBSV-inoculated plant. On 7 dpi on TBSV - infected plants appear twisting and spotting of apical leaves, growth retardation compared to control plants. By 21-28 dpi, TBSV-infected plants undergo systemic collapse.

Viral protein accumulation

Determination of viral infection was carried out for the presence of viral proteins in infected plants. Viral protein accumulation was analyzed by Western blot assay of P19 protein. The suppressor protein P19 is an indicator of a viral infection. Western blot analysis was performed using

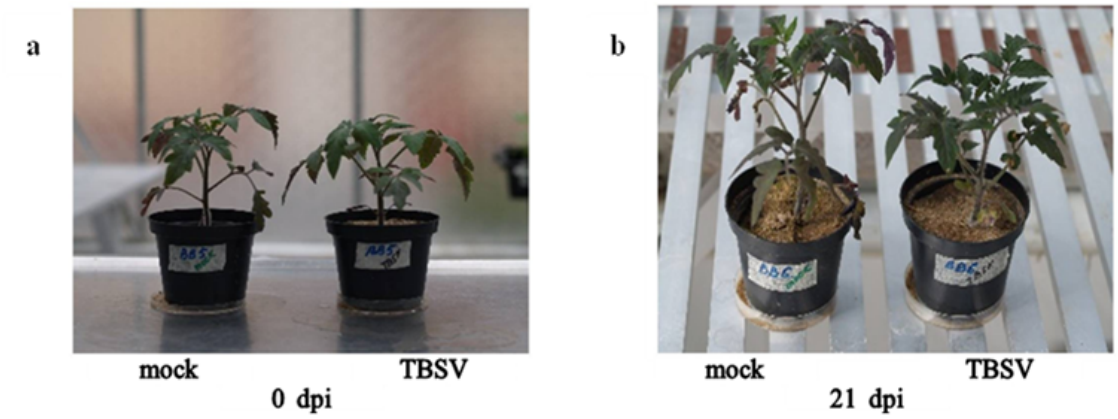

FIGURE 1 – **Symptom development of TBSV infection on tomatoes cv. *Better boy*: (a) Plants before inoculation. (b) Plants after 21 days post inoculation (dpi). In both figures on the left the control mock-inoculated plants, on the right the TBSV – infected plants. No visual symptoms of TBSV infection were observed during 21 dpi.**

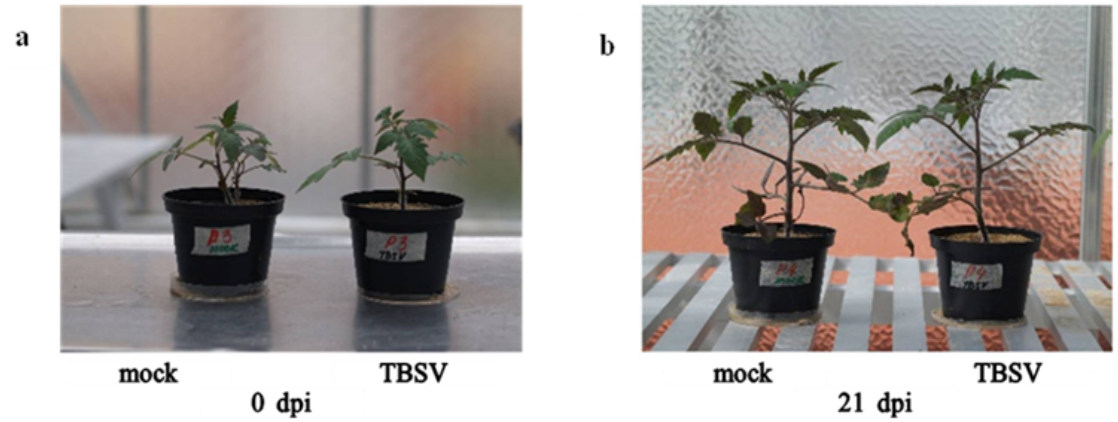

FIGURE 2 – **Symptom development of TBSV infection on tomatoes cv. *Pera*: (a) Plants before inoculation. (b) Plants after 21 days post inoculation (dpi). In both figures on the left the control mock-inoculated plants, on the right the TBSV – infected plants. No visual symptoms of TBSV infection were observed during 21 dpi.**

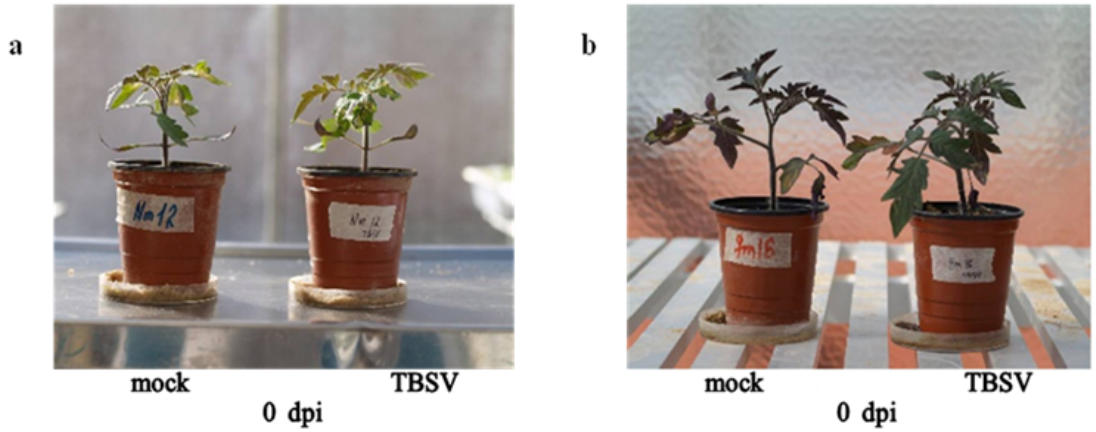

FIGURE 3 – **Symptom development of TBSV infection on tomatoes cv. Money Maker: (a) Plants before inoculation. (b) Plants after 21 days post inoculation (dpi). In both figures on the left the control mock-inoculated plants, on the right the TBSV – infected plants. No visual symptoms of TBSV infection were observed during 21 dpi.**

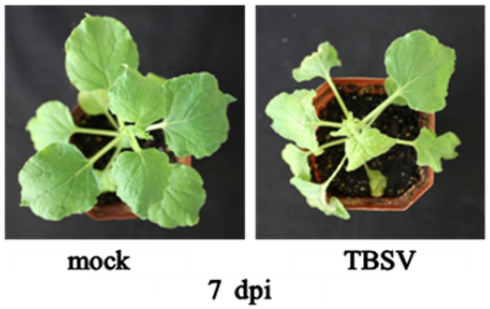

Figure 4 – **Symptom development of TBSV infection on *N. Benthamiana* at 7 dpi.**

polyclonal antibodies against P19 (Fig. 5). Apical, non-inoculated leaves of tomatoes were used for an experiment.

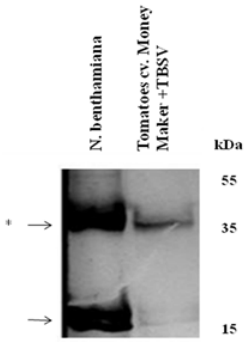

Figure 5 – **Western blot analysis of TBSV infected plants.**

Western blot analysis revealed presence of P19 protein in samples extracted from leaves of *N. benthamiana* and in tomato plans cv. *Money Maker*. However the amount of P19 protein was lower in *Money Maker* plants than in *N. benthamiana*. The expression of the viral protein was not detected in tomatoes cv. *Pera* and *Better Boy* (results not shown). This indicates that despite the absence of visual symptoms the inoculation of cv. *Money Maker* resulted in successful accumulation of the virus. Rabbit P19 antiserum was used for Western blot protein detection, and immune complexes were visualized using alkaline phosphatase hydrolysis of tetrazolium-5-bromo-4-chloro-3-indolyl phosphate as the substrate. Molecular mass markers in kDa are indicated on the right side of the panel. *- dimer of P19 protein observed after SDS-PAGE.

Effect of TBSV infection on the photosynthesis of *N. benthamiana* and tomato cv. *Money maker*

To determine the effect of TBSV infection on the photosynthesis parameters of *N. benthamiana* and tomato cv. *Money maker*, the leaves of 30 days old plants were mechanically inoculated. During the entire experimental period of 30 dpi, control and TBSV infected plants were analyzed at 3, 7, 14 and 21 dpi. The level of photosynthesis was measured using a portable digital fluorometer FP100. The fluorometer allows a rapid and accurate measurement of chlorophyll fluorescence parameters. Measurement of the activity of the photosynthesis and photographic analysis were performed every 3 days after the inoculation on the middle and apical leaves, in order to detect the exact period when the effect of the infection would be the most pronounced even if there are no visible symptoms. A combined graph was being plotted for the mean values of the fluorescence intensity of chlorophylls after every measurement of the photosynthesis activity of 40 plants (Fig 6, 7).

The diagram shows the results of the average means of the photosynthesis level on the apical (non-inoculated) leaves of *N. benthamiana* and tomatoes cv. *Money maker* at different days post inoculation (dpi): where "ns" is a non-significant difference between the numeric data, Student's t-test. According to the data obtained by the digital fluorometer, there are no significant differences between control and infected plants in the level of photosynthesis during 14 dpi for *N.benthamiana*

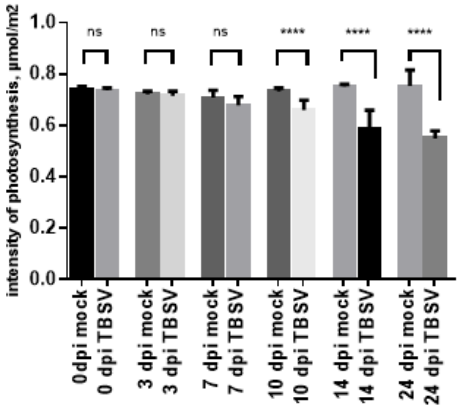

Figure 6 – **The average level of plant photosynthesis on the apical leaves of *N. benthamiana* for 24 days of the experimental period.**

and after 14 dpi the level of photosynthesis sharply decreases in the infected plants, whereas in the control plants there are no significant changes during the entire experimental period (24 dpi).

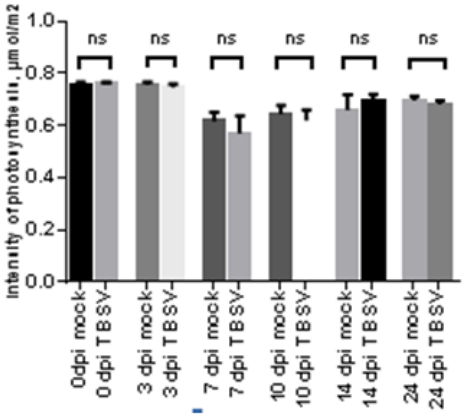

Figure 7 – **The average level of plant photosynthesis on apical leaves of *Money maker* for 24 days of the experimental period.**

The diagram shows the results of the average means of the photosynthesis level on the apical (non-inoculated) leaves of *N. benthamiana* and tomatoes cv. *Money maker* at different days post inoculation (dpi): where "ns" is a non-significant difference between the numeric data, Student's t-test. According to the data obtained by the digital fluorometer, for the tomato cv. *Money maker*, the difference between the levels of photosynthesis in both infected and control plants was non-significant during all experimental period.

In addition to this analysis, Chl-FI was also performed. The experiment was carried out in two biologic repeats on 40 plants in each of them (Fig.8, 9). The kinetics of the pathogenesis of TBSV in *N. benthamiana* and the *Money maker* was monitored by registering images using various Chl-FI parameters and comparing control and infected plants. The photosynthetic parameters were measured every 3 days for 21 dpi. In the first 7 days, there were no changes that differed from the control plants. The first changes were registered during the period between 7-14 dpi for *N. benthamiana* plants (Fig.8), and for *Money maker* during the period from 14-21 dpi (Fig.9).

After conducting Chl-FI studies, it was found that the leaves of the mock-inoculated plants fluorescence in intense blue color, indicating a sufficient amount of chlorophylls in the leaves. And also slightly red color indicates the presence of secondary metabolites, which is the norm for healthy plants, but under biotic stress conditions the level of secondary metabolites rises because of the

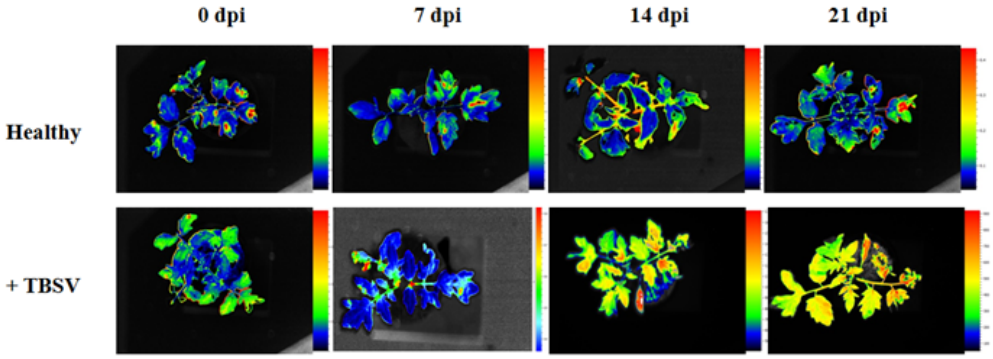

FIGURE 8 – **Chl-FI imaging of *N. benthamiana*. Blue color indicates a sufficient amount of chlorophylls in the leaves, red color indicates the presence of secondary metabolites. Fluorescence Imaging system (Chl-FI), allows to observe the red fluorescence emitted by chlorophyll *a* (Chl *a*), poses the possibility of studying the change in the efficiency of photosynthesis in leaves in response to the biotic and abiotic factors. A Chl-FI study showed the presence of an intense blue fluorescence and a light red colour in the leaves of the control plants. In the leaves of TBSV-infected plants, an intense red light was detected, which increased starting from 7 dpi for *N. benthamiana***

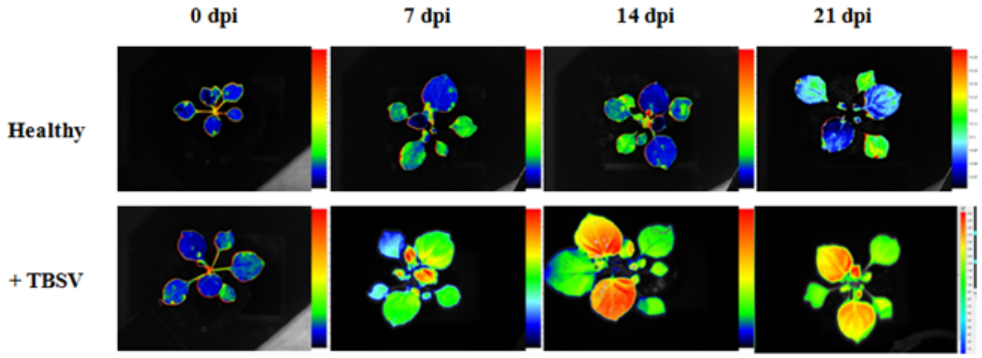

FIGURE 9 – **Chl-FI imaging of tomatoes cv. *Money maker*.Blue color indicates a sufficient amount of chlorophylls in the leaves, red color indicates the presence of secondary metabolites. Fluorescence Imaging system (Chl-FI), allows to observe the red fluorescence emitted by chlorophyll *a* (Chl *a*), poses the possibility of studying the change in the efficiency of photosynthesis in leaves in response to the biotic and abiotic factors. A Chl-FI study showed the presence of an intense blue fluorescence and a light red colour in the leaves of the control plants. In the leaves of TBSV-infected plants, an intense red light was detected, which increased starting from 14 dpi for tomatoes cv. *Money maker***

malfunction of the photosystems. Therefore, an intense red fluorescence was detected in the infected samples [22]. A Chl-FI study showed the presence of an intense blue fluorescence and a light red colour in the leaves of the control plants. In the leaves of TBSV-infected plants, an intense red light was detected, which increased starting from 7 dpi for *N. benthamiana* and from 14 dpi for tomatoes cv. *Money maker*.

Discussion. Viral infection is one of the biotic stresses that reduces yield of crops. Plants may differently response to viral infection. Four plant categories are distinguished based on the degree of resistance for viral infection: sensitive, tolerant, supersensitive and extremely resistant [23].

When we inoculated 3 different cultivars of tomato with TBSV virus, we found that not all the cultivars show similar symptoms to the TBSV infection (Fig1,2,3).

Viruses production of the diagnosis of a viral infection by western blotting assay of the viral protein P19 was carried out in order to determine a category of above-mentioned cultivars of tomatoes.

The results of inoculation of three different tomato cultivars demonstrated that the cv. *Money maker* is tolerant to TBSV infection, as it showed an accumulation of TBSV P19 with no visible symptoms (Fig. 3, 5). While in the cultivars *Pera* and *Better boy* neither an accumulation of P19 protein (Fig.5) nor symptoms were detected, which indicates their resistance to TBSV infection (Fig.1, 2). This implies the presence of certain protection mechanisms within species [24] .

According to the data obtained by the digital fluorometer, for the tomato cv. *Money maker*, the difference between the level of photosynthesis in both infected and control plants was non-significant (Fig.7), but for *N.benthamiana* (Fig.6), and after 14 dpi the level of photosynthesis sharply decreases in the infected plants, whereas in the control plants there are no significant changes during the entire experimental period (24 dpi). Thus it can be concluded that the reduction of the level of photosynthesis in TBSV-infected *N. benthamiana* after 14 dpi is probably due to an impaired function of the chloroplasts [25].

Data obtained by Balachandran S. et al. (1997) showed that the early activation of the protective mechanisms of *N. tabacum* against the infection of the tobacco mosaic virus (TMV) leads to local damages of the photosynthetic apparatus, which in turn results in the impairment of photosynthetic parameters [26]. Moreover, the viral infection inhibits the expression of genes associated with the function of chloroplasts and photosynthesis [27].

Due to the fact that in the previous experiment on *Money maker* there were no visible symptoms and changes in the level of photosynthesis in TBSV-infected tomatoes (Fig. 7), while the P19 virus protein was expressing (Fig. 5), it was decided to study other parameters of the photosynthesis. Therefore, Chlorophyll Fluorescence Imaging system (Chl-FI) was used to study the effect of *TBSV* on *N. benthamiana* and cv. *Money maker*. This equipment is especially used in the case of biotic stress, when visible symptoms, the heterogeneity of metabolism and the activity of photosynthesis cannot be detected [28] as our plants showed changes in 14 days. The reason might be change in the structure and function of the chloroplast that leads to chlorosis and leaf necrosis by damaging Calvin cycle and carbohydrate metabolism as a result of viral infection [29]. The development of the technology for the processing of various images, such as the Chlorophyll (indeed similarly, Fig.8 and Fig. 9) [30].

Conclusion. The degree of TBSV infection varies depending on the cultivar of the tomatoes: the tomatoes *Better boy* and *Pera* are resistant, and the *Money maker* is tolerant to the TBSV infection, because even with the accumulation of viral proteins plants do not get sick and therefore do not show visible symptoms of the virus infection. These results show that the plants might contain a number of resistance genes that selectively response to the same virus. The analysis carried out using the Chlorophyll Fluorescence Imaging System (Ch-FI) and fluorometer demonstrated that despite the absence of symptoms in TBSV-infected *Money maker* tomatoes, there are the increase in the level of secondary metabolites and a slight reduction of the photosynthesis activity.

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Сорт - специфическое влияние инфекции TBSV вируса на томаты

Аннотация: В данном исследовании изучено влияние инфекции вируса кустистой карликовости томатов (TBSV) на три сорта *Solanum lycopersicum (томаты)* и модельное растение *Nicotiana benthamiana.* Согласно нашим исследованиям, степень заражения TBSV варьируется в зависимости от сорта томатов. Сорта *Better boy* и *Pera* устойчивы, в то время как сорт *Money maker* был толерантен к инфекции *TBSV*. Эти результаты показывают, что растения могут содержать различные гены устойчивости, которые избирательно реагируют на вирус TBSV и указывают на наличие определенных защитных механизмов внутри вида. Кроме того, анализ, проведенный с использованием системы флуоресцентной визуализации хлорофилла (Chl-FI) и цифрового флюорометра, показал, что, несмотря на отсутствие визуальных симптомов инфекции у сортов томатов, наблюдается повышение уровня вторичных метаболитов и незначительное снижение активности фотосинтеза.

Ключевые слова: Chlorophyll fluorescence imaging, фотосинтез, TBSV*, Solanum lycopersicum copm Money Maker, Nicotiana benthamiana.*

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TBSV вирус инфекциясының қызанақ сұрыпына өзіндік әсері

Аннотация: Бұл зерттеуде біз қызанақтың бұталы ергежейлілік вирусының (TBSV) Solanum lycopersicum (томаттар) және Nicotiana benthamiana модельдік өсімдігіне әсерін зерттеген. Біздің зерттеулерге сәйкес, TBSV-ң инфекциялау дәрежесі қызанақ сұрыбына байланысты өзгереді. Better boy және Pera сұрыптары TBSV инфекциялауға тұрақты, ал Money maker сұрыбы TBSV инфекциялауға төзімді болды. Бұл нәтижелер өсімдіктерде TBSV вирусына қарсы таңдамалы төзімділіктің бар екендігін және түр ішінде белгілі бір қорғаныс механизмдерінің болуын көрсететін әртүрлі тұрақтылық гендері болуы мүмкін екенін көрсетеді. Бұдан басқа, хлорофиллді (Chl-FI) флуоресцентті визуализациялау және сандық флюорометр жүйесін пайдалана отырып жүргізілген талдау қызанақ сұрыптарында инфекцияның визуалды симптомдарының болмауына қарамастан, екінші реттік метаболиттер деңгейінің жоғарылауы және фотосинтез белсенділігінің шамалы төмендеуі байқалғанын көрсетті.

Түйін сөздер: Chlorophyll fluorescence imaging, фотосинтез, TBSV, Solanum lycopersicum Money Maker сұрыбы, Nicotiana benthamiana.

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