SPECTRAL REFLECTANCE TECHNIQUE FOR DETECTION OF VIRAL INFECTIONS IN TOMATO PLANTS (*LYCOPERSICON ESCULENTUM L.)*

Dora Krezhova[1], Dimitrinka Hristova[2] , Tony Yanev[1]

[1]Space and Solar-Terrestrial Research Institute – Bulgarian Academy of Sciences
[2]Plant Protection Institute - Ministry of Agriculture and Food
e-mail: dkrezhova@stil.bas.bg

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Abstract: *Spectral reflectance technique was applied to assess the influence of viral infection and some growth regulators on tomato plants (Lycopersicon esculentum L.) inoculated with Tomato mosaic virus (ToMV). Spectral measurements were provided on young plants, Newton cultivar, resistant to ToMV. The plants were grown in a greenhouse under controlled conditions. Leaf reflectance was collected by a portable USB2000 fibre-optic spectrometer in the visible and near infrared spectral ranges (450-850 nm). The analyses were performed on six groups of plants: healthy (control), inoculated with ToMV, and treated with four growth regulators - Spermine preparation; MEIA (beta-monomethyl ester of itaconic acid); BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester) and Phytoxin VS, and then inoculated with ToMV. Comparative analysis of the results from the leaf spectral reflectance and viral concentrations in the leaves determined by the Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) serological method was carried out. The statistical significance of the changes in the leaf reflectance spectra of treated plants against the control was established using the Student's t-criterion.*

ОТРАЗЕНА СПЕКТРАЛНА РАДИАЦИЯ КАТО МЕТОД ЗА ОТКРИВАНЕ НА ВИРУСНИ ИНФЕКЦИИ В ДОМАТЕНИ РАСТЕНИЯ (L*YCOPERSICON ESCULENTUM L.)*

Дора Крежова[1], Димитринка Христова[2], Тони Янев[1]

[1]Институт за космически и слънчево-земни изследвания - Българска академия на науките
[2]Институт за защита на растенията - Министерство на селското стопанство и храните
e-mail: dkrezhova@stil.bas.bg

Резюме: *Дистанционният метод на отразена радиация е приложен за оценка на влиянието на вирусни инфекции и на някои растежни регулатори върху доматени растения (Lycopersicon esculentum L.) заразени с вируса на доматената мозайка (ToMV). Спектралните измервания са проведени върху млади растения от сорт Нютон, който е устойчив на ToMV. Растенията са отгледани в оранжерия при контролирани условия. Отразената от листата радиация е получена с преносим спектрометър с влакнеста оптика USB2000 във видимата и близката инфрачервена спектрални области (450-850 nm). Анализите са извършени върху шест групи растения: здрави (контрола), заразени с ToMV, и обработени с четири регулатори на растежа - препаратите Spermine; MEIA (beta-monomethyl ester of itaconic acid); BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester) и Phytoxin VS, а след това заразени с ToMV. Проведен е сравнителен анализ на резултатите, получени по метода на спектралното отражение и вирусните концентрации в листата, определени по серологичния метод DAS-ELISA за анализ и доказване на растителни вируси. Статистическата значимост на промените в спектрите на отражение на листа от третираните растения спрямо контролната група е определена чрез използване на критерия на Стюдънт.*

Introduction

Great economic losses occur worldwide due to the viral plant diseases. Losses are often more insidious, frequently less conspicuous and therefore go unnoticed and untreated. The tomato *(Lycopersicon esculentum L.)* is one of the most widely grown vegetable food crops in the world [1].

Because of its importance as food, tomato has been bred to improve productivity, fruit quality, and resistance to biotic and abiotic stresses. Tomato has been widely used not only as food, but also as research material. The tomato plant has many interesting features such as fleshy fruit, a sympodial shoot, and compound leaves, which other model plants (e.g., rice and Arabidopsis) do not have [2]. Tomato is considered a heavy feeder of micronutrients and boron in particular is important for its growth, fruit set, and disease resistance [3]. Damage to tomato plants is especially important in case of virus transmission.

Diseases caused by Tomato mosaic virus (ToMV) are among the most important factors limiting not only tomato production but they can cause epidemics in many crops and completely destroy the yield [4]. The virus is seed-borne. Infested tomato seeds can be the source of infection and the means by which the virus can be disseminated over large distances. Only a few seedlings need to be infected for the rapid spreading of the virus. Symptoms can be found during any growth stage and all plant parts are affected. Generally, infected with ToMV plants have a light or dark green mottling or mosaic with distortion of younger leaves, and stunting to varying degrees (Fig. 1). Symptoms are influenced by environmental conditions such as daylength, temperature, and light intensity as well as by variety, plant age at infection, and virulence of ToMV strain. The virus is quite stable under adverse environmental conditions and can persist in plant debris in dry soil for 2 years or in moist soil for 1 month or in root debris in fallow soil for 22 months. It can also persist in greenhouse structures for long periods of time [5]. On susceptible cultivars, symptoms may range from severe to none.

Various synthetic and biological compounds have been described of being capable of controlling a large variety of plant diseases without displaying a direct antibiotic effect themselves. These substances are called inducers, based on their ability to induce resistance in the treated plans. Both biologically as well as chemically induced resistance against pathogen attack have been described for many plant species against a wide variety of pathogens ranging from oomycetes, fungi, bacteria to viruses [6-8]. Both types of induction share similarities at the phenotypic level, such as a hypersensitive response, trailing necroses, wall strengthening in form of papillae and lignification, and at the molecular level, where a similar set of genes has been observed to be induced. These genes are termed SAR (systemic acquired resistance) genes and their expression depends on salicylic acid (SA). Chemical inducers of resistance seem to enter at different points in defence pathways. The most thoroughly investigated chemical inducers are those interfering with the SA pathway, such as growth regulators BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester), MEIA (beta-monomethyl ester of itaconic acid), Spermine (derivative of putrescine (1,4-diaminobutane)) and Phytoxin SV. They have been described as having effect on various plant diseases because they can induce SAR without the plant-pathogen interaction [9, 10].

Remote sensing techniques from ground to satellite platform have proven to be very effective to monitor insect infestation and disease epidemic in agricultural crops and other plants within a given growth condition [11]. Many studies have shown that the basis for distinguishing healthy and stressed plants using optical remote sensing technique is their differences in reflectance in different spectral regions [12-14]. Healthy plant often has a high reflectance in the near-infrared (NIR) region determined by cellular and subcellular structures and a low visible (VIS) reflectance due to strong pigment absorption [15]. Changes in pigment concentrations as well as internal leaf structure are strongly related to the physiological status, and consequently, spectral features of plant [12, 16]. Leaf spectral reflectance differences between healthy and diseased plants give a possibility for early, efficient and objective confirmation of health and evaluation of plant responses to different stress factors and diseases. The detection of viruses in plants involves destructive sampling followed by testing by enzyme-linked immunosorbent assay (ELISA) and/or reverse transcription-polymerase chain reaction (RT-PCR).

In this study, we have investigated the responses of tomato plants *(Lycopersicon esculentum L.),* cv. Newton, to infection by Tomato mosaic virus and the influence of resistance inducers applied over the infected plants by making use of a non-invasive remote sensing technique, spectral reflectance measurements, in the VIS and NIR spectral ranges. The degree of the infection and its incidence to plants were determined by the serological method *Double antibody sandwich enzyme-linked immunosorbent assay* (DAS-ELISA) by which the concentration of the ToMV was assessed. Four resistance inducers (BTH, MEIA, Spermine and Phytoxin VS) were examined for their efficacy in controlling the viral infection in tomato plants.

Plant material

Tomato plants *(Lycopersicon esculentum L.)* were grown in a greenhouse under control conditions (temperature 24-26^{o}C and 16 h light/ 8 h dark photoperiod). The seeds were sowed and after germination were partitioned into six groups. The plants from the first group were not treated (control plants). The plants from the second group at stage 4-6 expanded leaf were inoculated with ToMV by following the Noordam method [17]. As infection source tomato leaves of cultivar Ideal infected by race 0 of ToMV were used. The third group was treated by preparation Phytoxin VS at concentration 0.25 %. After the tomatoes were pulverized three times every ten days, they were inoculated by ToMV as the plants of the second group. The rest three groups were treated just a single time with resistance inducers: Spermine, MEIA and BTH. On the next day inoculation with ToMV according to Noordam was made. Fig. 2 shows some of the investigated leaves from the first three groups of plants – control, inoculated with ToMV and treated with preparation Spermine.

Fig.1. Tomato mosaic virus on greenhouse plants

Fig. 2. Investigated tomato leaves from healthy (1), ToMV (2) and Spermine (3) groups

Methods

Leaf Spectral Reflectance

Spectral reflectance was collected by using an Ocean Optics USB2000 spectrometer in the VIS and NIR spectral ranges (450-850 nm) at 1170 spectral wavebands [18]. As a source of light a halogen spectral lamp, providing homogeneous illumination of the measured areas was used. The spectral reflectance characteristics (SRC) were obtained as a ratio of the intensity of leaf reflected light to the light reflected from a diffuse reflectance standard for each wavelength in the interval. Spectral measurements were performed on 7^{th} and 14^{th} day after the inoculation with ToMV on by twenty detached leaves as from uninfected as well as from infected plants.

Serological method

The viral concentrations in the plants were determined by the serological method Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The samples for DAS-ELISA were taken 14 days after the inoculation. The analysis was made using a ELISA kit for ToMV, LOEVE (Biochemica GmbH Sauerlash, Germany). The reaction was recorded by a spectrophotometer ELISA reader type Anthers 2010 at a wavelength of 405 nm. The effect of the used resistance inducers on the infection progress was assessed.

Statistical method

The Student's t-criterion was applied for analysis of the statistically significant differences at $p<0.05$ between the means of values of the reflectance spectra of uninfected (control) and virus-infected leaves at eight wavelengths: λ_1 = 524.29 nm, λ_2 = 539.65 nm, λ_3 = 552.82 nm, λ_4 = 667.33 nm, λ_5 = 703.56 nm, λ_6 = 719.31 nm, λ_7 = 724.31 nm, and λ_8 = 758.39 nm, disposed uniformly over the investigated spectral intervals.

Results and discussion

The average SRC over all measured areas of the six groups of tomato plants on the 7^{th} day after the inoculation with ToMV are shown in Fig. 3. The analysis of reflectance data was performed in four spectral ranges: green (520-580 nm), red (640-680), red edge (690-720 nm) and NIR (720-780 nm) in which the differences between SRC were most significant.

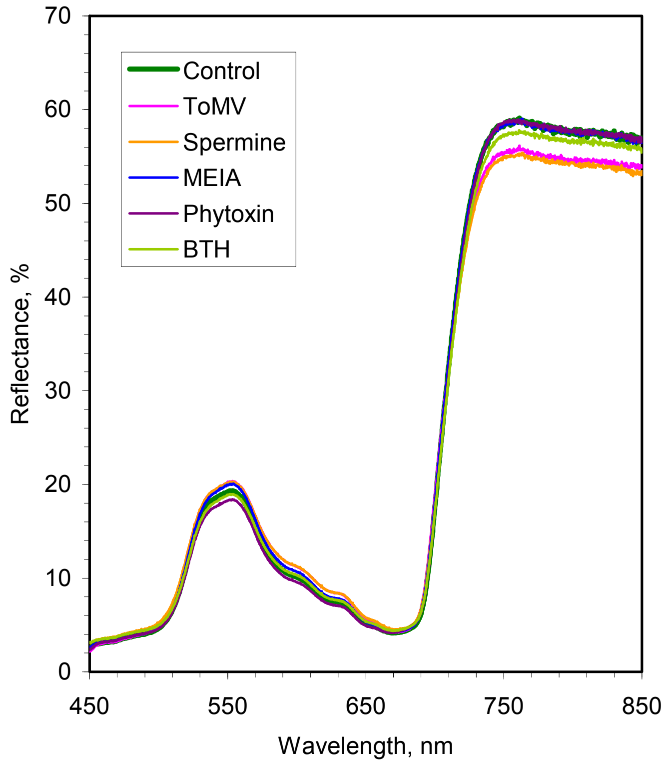

Fig. 3. Averaged SRC of six groups of tomato plants on the 7th day after the inoculation with ToMV

It is observed that the averaged SRC for the six groups of plants differed insignificant. Differences were manifested in the ranges of maximal chlorophyll reflectance to minimal absorbance and NIR. The increase of SRC values with respect to control in the cases of treatment with ToMV and the growth regulators Spermine and MEIA was fined in spectral range 500-650 nm while in the NIR SRC values decreased. These changes were due to the presence of a little quantity of viruses in the leaves what caused the reduction of pigment content, the chlorophyll content decreased. The effect of the other two growth regulators on the spectral properties of the leaves was the opposite. The leaves were more dark green then control and the chlorophyll content increased.

The results of application of the Student's t-criterion for the data collected on the 7th day after the inoculation with ToMV are displayed in Table 1. Statistically significant differences between data pairs of the control and infected plants at $p<0.05$ were established only in single wavelengths for all cases.

Table 1. p-values of the Student's t-criterion for pairs of the control and infected plants on the 7th day after the inoculation

Pairs	Control	ToMV		Spermine		MEIA		BTH	Phytoxin VS		
compared	mean	p	mean	p	mean	p	mean	p	mean	p	mean
λ_1 / λ_{1c}	13.67	0.033	14.60	0.040	14.65	0.236	14.09	0.678	13.53	0.060	13.00
λ_2 / λ_{2c}	18.36	0.079	19.24	0.069	19.32	0.114	19.03	0.485	18.08	0.033	17.48
λ_3 / λ_{3c}	19.29	0.067	20.27	0.089	20.27	0,095	20.04	0.542	19.03	0.025	18.33
λ_4 / λ_{4c}	4.19	0.502	4.29	0.002	4.60	0.644	4.25	0.038	4.45	0.916	4.18
λ_5 / λ_{5c}	23.26	0.033	24.17	0.796	23.23	0.384	23.48	0.082	22.30	0.084	22.29
λ_6 / λ_{6c}	44.26	0.127	43.33	0.003	42.33	0.919	44.32	0.007	42.83	0.197	43.46
λ_7 / λ_{7c}	48.96	0.020	47.44	0.001	46.72	0.574	49.26	0.023	47.70	0.297	48.26
λ_8 / λ_{8c}	58.72	0.000	55.64	0.000	55.04	0.749	58.53	0.084	57.74	0.965	58.69

The averaged SRC of the six groups of tomato plants on the 14th day after the inoculation with ToMV are shown in Fig. 4.

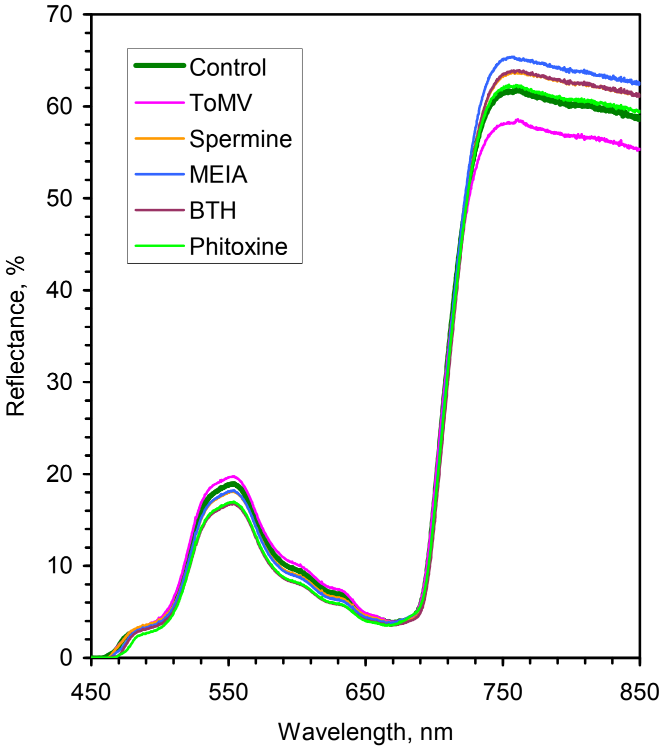

Fig. 4. Averaged SRC of six groups of tomato plants on the 14th day after the inoculation with ToMV

SRC of inoculated only with ToMV leaves shows again slightly higher values than control in the range 500-650 nm. This is because of the reduction of the green colour of the leaves. Visual changes for presence of the viral infection on the plants were not detected. The rest SRC are with reduced values as compared with control because of normalized growth owing to the applied preparations. The results from the Student's t-criterion for pairs of the control and infected plants are presented in Table 2.

Table 2. p-values of the Student's t-criterion for pairs of the control and infected plants on the 14th day after the inoculation

Pairs	Control	ToMV		Spermine		MEIA		BTH	Phytoxin VS		
compared	mean	p	mean	p	mean	p	mean	p	mean	p	mean
λ_1 / λ_{1c}	13.04	0.060	14.01	0.003	12.32	0.003	12.32	0.000	11.23	0.000	11.42
λ_2 / λ_{2c}	17.96	0.072	18.85	0.003	17.14	0.001	17.19	0.000	15.84	0.000	16.03
λ_3 / λ_{3c}	18.90	0.009	19.7	0.003	18.07	0,013	18.13	0.000	16.73	0.000	16.92
λ_4 / λ_{4c}	3.87	0.399	3.95	0.782	3.84	0.501	3.79	0.007	3.55	0.003	3.52
λ_5 / λ_{5c}	22.50	0.070	23.4	0.000	20.61	0.004	21.48	0.000	19.72	0.000	21.1
λ_6 / λ_{6c}	45.36	0.085	44.5	0.037	44.34	0.45	45.77	0.000	43.73	0.062	44.4
λ_7 / λ_{7c}	50.72	0.006	49.14	0.564	50.38	0.042	51.96	0.099	50.00	0.355	50.19
λ_8 / λ_{8c}	61.57	0.000	58.24	0.017	63.73	0.000	65.26	0.000	63.88	0.452	62.14

Statistically significant differences between the mean values of the data of control and plants treated only with ToMV were established at λ_3 and NIR region. Differences for SRC of plants sprayed with preparations Spermine, MEIA, BHT and Phytoxin VS against the control were significant for most wavelengths. The highest differences between SRC were found in the cases of treatment with BTH and MEIA.

The results from DAS-ELISA analysis on the 14th day post inoculation are displayed in Fig. 5. Extinction values do not show presence of viral infection from all six groups of plants which is a proof that the cultivar Newton is resistant to ToMV. They are near to the control (0.150 OD, optical density). Most higher are for the plants treated with growth regulators BTH and MEIA.

In summary it was found that the tomato plants, cv. Newton, were not infected with ToMV. On the 14th day the differences between SRC were statistically significant in more wavelengths than on the 7th day in the cases of treatments with the four resistance inducers owing to the stimulating effect on their growth.

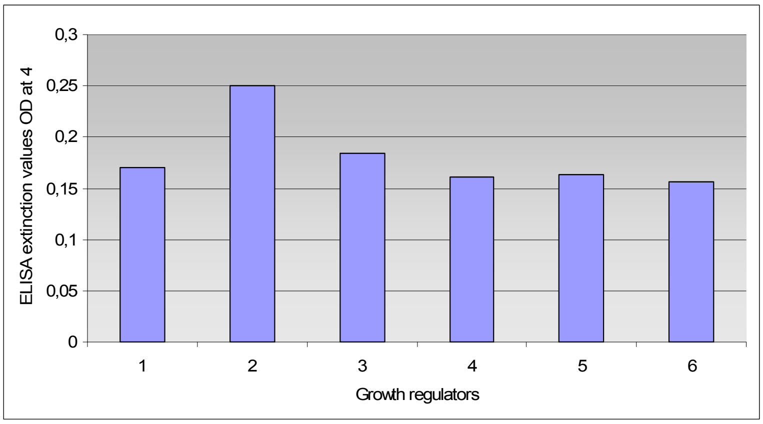

Fig. 5. Influence of growth regulators at ToMV in tomato plants, cv. Nuton: 1 - Spermine, 2 - BTH, 3 - MEIA, 4 - Phytoxin, 5 - ToMV, 6 – control

The correlation between the DAS-ELISA extinction values and changes found by spectral reflectance technique indicate that this remote sensing method is sensitive, reliable and suitable for early diagnostics of virus infection.

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