Chlorophyll Fluorescence Analysis of Tomato Plants *(Lycopersicon esculentum L.)* Infected with Tomato Mosaic Virus (ToMV)

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Abstract. The chlorophyll fluorescence technique was applied to evaluate the influence of tomato mosaic virus (ToMV) on leaves of young tomato plants (*Lycopersicon esculentum L.*), cultivar Ideal sensitive to this virus. Four commercially available growth regulators were used with the aim to assess their ability to induce systemic acquired resistance. The plants were grown in a greenhouse under controlled conditions. Part of them was regarded as a control (healthy). The rest part was divided into five groups. At growth stage 4-6 expanded leaf, the plants from the first group were infected with ToMV. The plants from the other groups were sprayed with the preparations Spermine, 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. The chlorophyll fluorescence was registered by a fibre-optics spectrometer (Ocean Optics) in the spectral range 600-900 nm. The measurements were performed on the 7th and 14th day after the viral infection. The Student's t-criterion was applied at five characteristic for the fluorescence spectra parameters. Statistically significant changes were established for the registered spectra for all cases of treatment against the control in the spectral range 650-740 nm with the exception of the spectra of Spermine treated plants. It was found that the chlorophyll fluorescence results correlate with the outcome from the spectral reflectance analyses and the serological analyses (DAS-ELISA) for presence of ToMV performed on the same plants and provide the possibility for express and reliable diagnosis of viral infections.

Keywords. Chlorophyll fluorescence, tomato mosaic virus (ToMV), systemic acquired resistance (SAR), Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), beta-monomethyl ester of itaconic acid (MEIA), Phytoxin VS, Spermine

Introduction

In nature plants are continually challenged by pathogens, such as fungi, bacteria, and viruses. However, only a few pathogens actually infect the plant and cause damage. Many plants defend themselves against fungi and other microbial pathogens by inducing both localized and systemic acquired resistance (SAR). Systemic acquired resistance (SAR) is an inducible defence mechanism that plays an important role in defending plants from attack by pathogens [1].

The application of safety chemicals to activate SAR-type reaction provides novel alternatives for disease control in agronomic systems. Recently the most utilized as growth regulators are the preparations Spermine, MEIA (beta-monomethyl ester of itaconic acid) and BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ester. Although these synthetic compounds activate plant defence mechanisms they do not have any direct activity against pathogens, and are hence classified as chemical activators or plant activators [2]. Plant activators are potentially very useful for crop disease control and management. However, their identification is challenging because they lack any direct toxic effects against pathogenic fungi and bacteria.

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Diseases caused by *Tomato mosaic virus* (ToMV) are among the most important factors limiting tomato production worldwide, as they can completely destroy the crop [3, 4]. Generally, infected with ToMV plants have a light or dark green mottling or mosaic with distortion of younger leaves, and stunting to varying degrees. Severely affected leaves may have a "fernlike" appearance and may show raised dark green areas. Fruit set may be severely reduced in affected plants [5].

The chlorophyll fluorescence is a sensitive indicator of the conversion of photosynthetic energy that occurs during the plant reaction to light. A small percent of ultraviolet and visible light absorbed by plant's pigments is re-emitted at longer wavelengths as fluorescence in blue, green, red and far-red bands. As this process is in competition with photosynthesis, the efficiency of the photochemistry of the plant, i.e., its physiological status, can be probed by means of chlorophyll fluorescence sensing, allowing to distinguish normal from stressed condition in intact plant material [6,7].

The main wavebands involved in the fluorescence emission from a green leaf when excited by UV-A radiation are in the blue at 440 nm, in the green at 520 nm, in the red at 690 nm and in the far red at 740 nm [8]. Those peaks in the red and far-red are primarily associated with chlorophyll-a fluorescence, which is directly related to the photosynthetic process, while the shorter wavelength emissions are predominantly associated with fluorescence from other, primarily phenolic, compounds in the leaves, especially from ferulic and chlorogenic acid bound to the cell walls. At any wavelength the fluorescence intensity is influenced by the concentration of the emitting substance, the internal optics of the leaf including factors that affect the partial re-absorption of the fluorescence, and especially for photosynthesis-related fluorescence the energy partitioning between the photosystems and the energy quenching processes in the chloroplast [9, 10]. The chlorophyll fluorescence has been proved as very useful tool in studies the stress responses of the plant leaves [11].

The investigations in this study were aimed to assess the potential of the leaf chlorophyll fluorescence in developing non-invasive techniques for real time disease diagnosis of young tomato plants infected with Tomato mosaic virus (ToMV) and to evaluate the presence of systemic acquired resistance induced in the plants at applied four growth regulators.

1. Materials and methods

1.1. Plant material and treatment

Greenhouse studies were conducted at the Plant Protection Institute, Kostinbrod, Bulgaria, with young tomato plants (*Lycopersicon esculentum L.*), cultivar Ideal sensitive to ToMV. The plants were grown under controlled conditions (temperature 24-26^{o}C, illumination 3500-4000 luxes and photoperiod 16/8 hours day and night). They were partitioned into six groups. The plants of the first group were healthy and untreated (control). The second group included plants inoculated at growth stage 4-6 expanded leaf with ToMV according to Noordam method [12]. The other four groups were sprayed with commercially available growth regulators: BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic acid-S-methyl ester), MEIA (beta-monomethyl ester of itaconic acid), polyamine Spermine and Phytoxin VS, and then inoculated with ToMV according [12].

1.2. Chlorophyll fluorescence

The spectral measurements of the leaf fluorescence were carried out in laboratory using a fibre-optics spectrometer USB2000 [13] in the spectral range 600-900 nm where the main part of the emitted from leaves fluorescence radiation is concentrated. The spectra were obtained in 915 wavebands with a spectral resolution 1.5 nm (halfwidth) in time-acquisition operating mode of the spectrometer. As a source of actinic light, a light diode LED 05B470Y-10C, emitting monochromatic light in the blue spectral range with light output maximum at 470 nm and light intensity of 507 µmol m^2 s^{-1} was used. Though the spectrum of exciting light is outside the spectral range 600-900 nm, a filter for yellow light is placed between the object studied and the spectrometer in order to discriminate the intensity of the transmitted exciting light. The tested leaves are dark adapted for 10 min before the measurements of fluorescence spectra. The abaxial side of the leaves is irradiated with actinic light and the exited fluorescence is measured from the adbaxial leaf surface.

1.3. Statistical analysis

All fluorescence spectra were normalized against the second maximum. Five parameters were defined to describe these normalized spectra: λ_1 - the wavelength at which the halfwidth of the spectra on the forefront is observed; λ_2 - the wavelength at which the first maximum is established; m - value of the fluorescence emission at λ= 667 nm at which wavelength the maximum of the relative differences is observed for all infected leaves; m_1 - amplitude of the first maximum of the fluorescence emission, m_2. - value of the fluorescence emission at λ_4 - wavelength at which the halfwidth of the spectra on the back front is observed. The Student's t-criterion was applied for analysis of the statistical significance of the differences between the means of parameters above derived from the fluorescence spectra. One normalized spectrum and the characteristic wavelengths and parameters chosen for statistical analysis are shown in Figure 1.

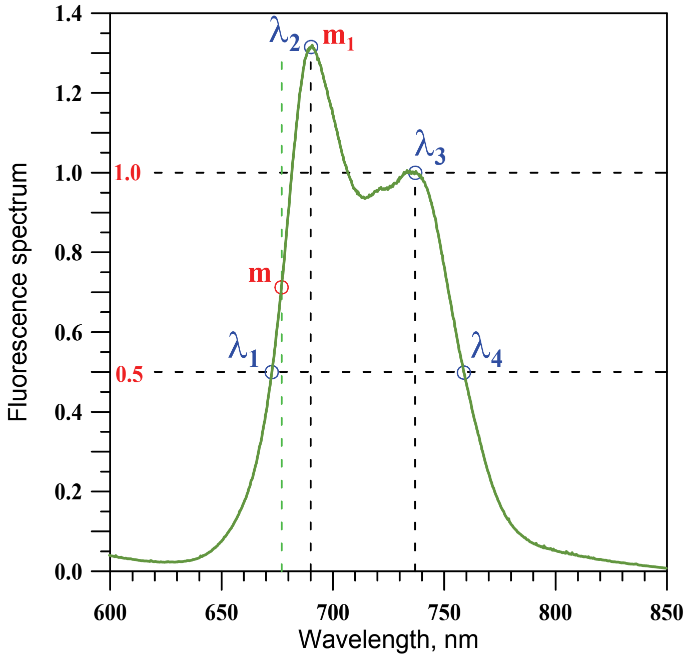

Figure 1. Normalized fluorescence spectrum and characteristic parameters for analysis

2. Results and discussion

The course of all fluorescence spectra of control plants in relative units (codes of the analog-to-digital converter) is shown in Figure 2.

The normalized spectra of the control group of plants against the second maximum at λ_3 = 737 nm are shown in Figure 3. The averaged fluorescence spectra over 20 control and 20 treated leaves from the five groups collected on the 7^{th} day after the inoculation with ToMV are shown in Figure 4. Changes in the spectra of treated plants against the controls were predominantly observed in the forefront and in the spectral range between first and second maximums (650-740 nm). The values of the spectra of the all treated plants decreased in this spectral range. The spectrum of the leaves sprayed with Spermine has values most close to the control spectrum.

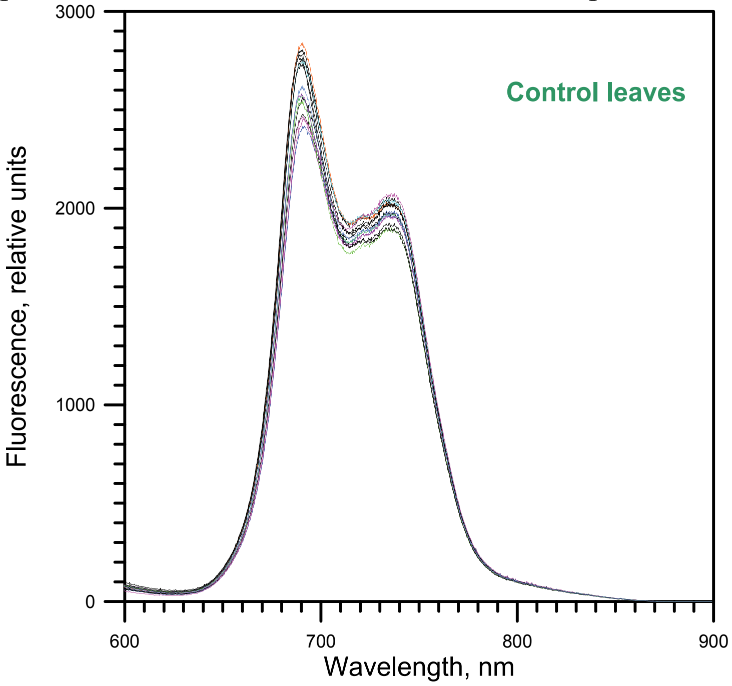

Figure 2. Fluorescence spectra of control tomato plants

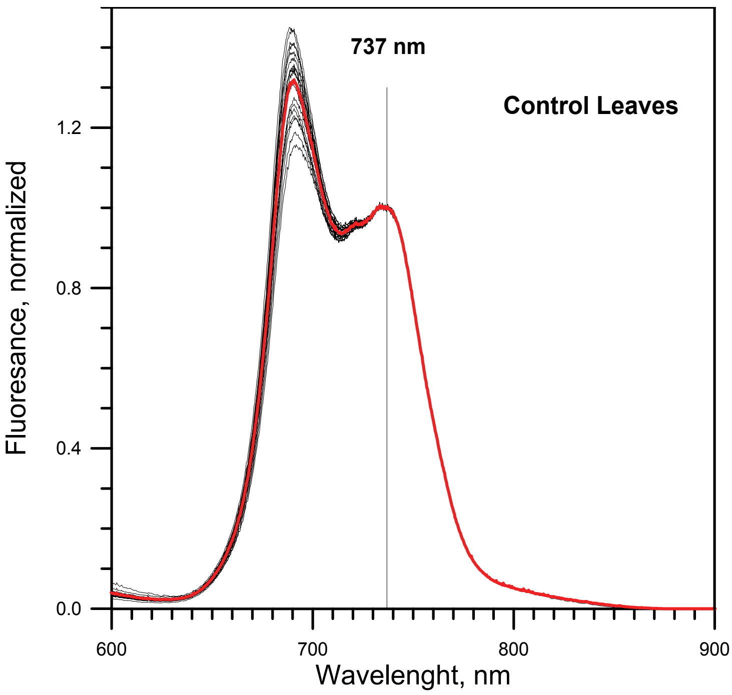

Figure 3. Normalized fluorescence spectra of control tomato plants

The normalized differences between the fluorescence spectra of the treated and control leaves were calculated by equation:

$$\Delta F(\lambda) = \frac{F_T(\lambda) - F_C(\lambda)}{F_T(\lambda) + F_C(\lambda)} \tag{1}$$

where: $F_C(\lambda)$ are the values of the averaged fluorescence spectrum of control plants at all 850 wavelengths (λ) and $F_T(\lambda)$ are the values of the averaged spectra of the treated plants for the same wavelengths.

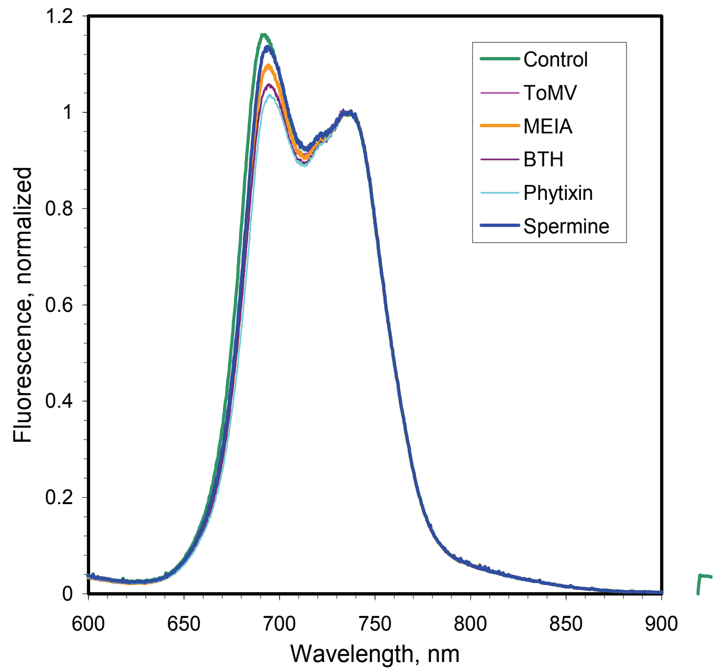

Figure 4. Averaged fluorescence spectra collected on the 7th day after the infection

Figure 5 shows the normalized spectral distribution of the differences in fluorescence of treated plants against the control in the spectral range 650-740 nm. The function ΔF(λ) for all infected cases exhibits a pronounced maximum at 677 nm (point **m** on the forefront, Figure 1). In the case of treatment with Spermine the differences were the smallest ones.

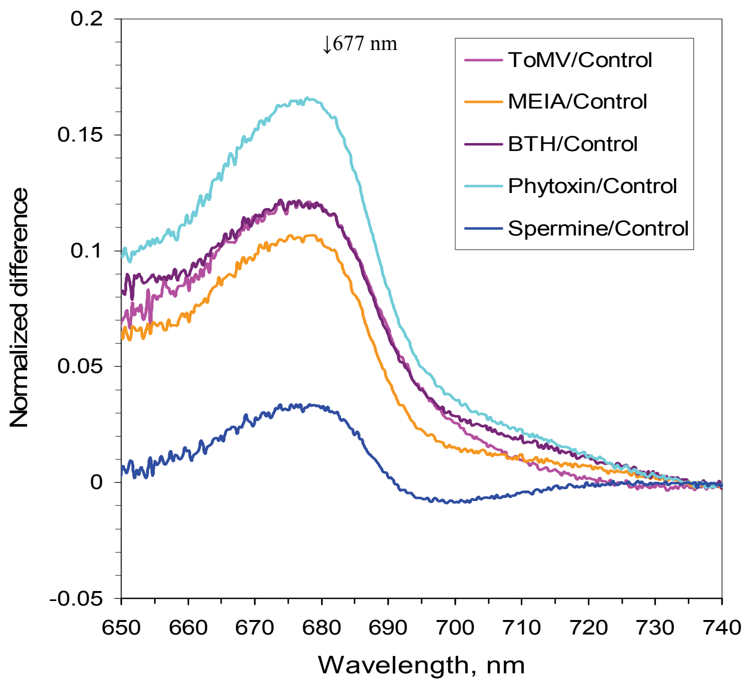

Figure 5. Normalized spectral distribution ΔF(λ) for data collected on the 7th day after the inoculation

Statistically significant differences were established at $p<0.05$ between the mean values of the data of the control and sprayed with preparations MEIA, BHT, Phytoxin VS, and treated only with ToMV plants for all parameters. No significant differences were established between the parameters m and λ_1 of control and sprayed with Spermine plants.

The averaged fluorescence spectra for the six investigated groups registered on the 14th day after carrying out the inoculation are shown in Figure 6. It is seen that the spectrum of Spermine sprayed leaves is most closely approaching the control spectrum.

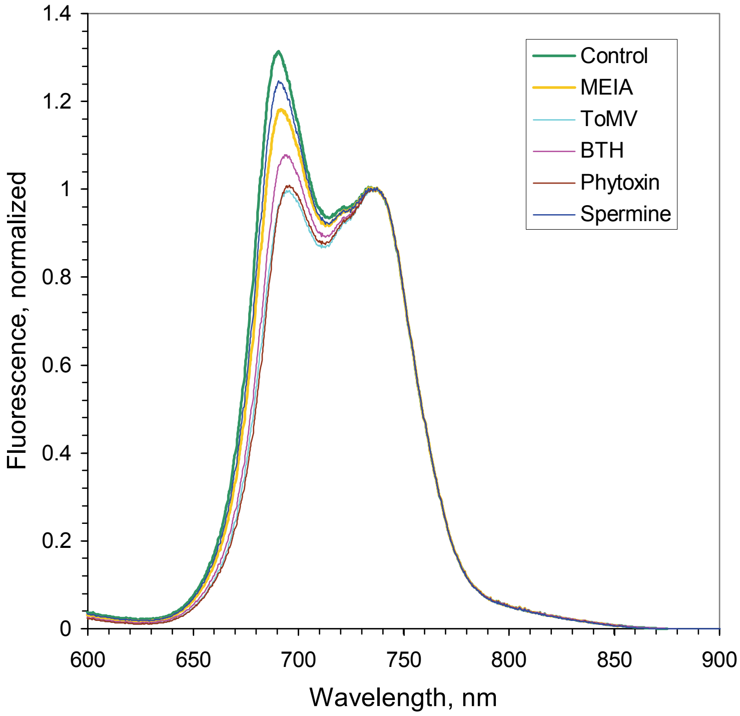

Figure 6. Averaged fluorescence spectra collected on the 14th day after the inoculation

The results for the significant p level of the Student's t-criterion for SRC measured on the 14th day after the inoculation with ToMV are set out in Tables 1a) and 1b).

Table 1a). p-values of the Student's t criterion for ToMV infection on the 14th day after the inoculation for cases ToMV, Spermine and MEIA

Pairs	ToMV		Spermine		MEIA	
compared	p	mean	p	mean	p	mean
λ_1 / λ_{1c}	0.000	674.93	0.010	673.74	0.000	679.0
λ_2 / λ_{2c}	0.000	692.37	0.040	691.40	0.000	695.2
m	0.000	0.586	0.061	0.649	0.000	0.367
m_1	0.000	1.181	0.056	1.26	0.000	1.015
m_2	0.033	0.504	0.708	0.502	0.504	0.504

Table 1b). p-values of the Student's t criterion for ToMV infection on the 14th day after the inoculation for cases BTH and Phytoxin VS and means of control

Pairs	Control	BTH		Phytoxin VS	
compared	mean	p	mean	p	mean
λ_1 / λ_{1c}	**mean**	0.000	677.83	0.000	643.84
λ_2 / λ_{2c}	672.56	0.000	694.24	0.000	695.51
m	690.75	0.000	0.466	0.000	0.504
m_1	0.712	0.000	1.112	0.000	1.01
m_2	1.322	0.129	0.503	0.000	0.395

In contrast with the results obtained on the 7th day after the inoculation, on the 14th day the SRC of control and treated leaves for the case Spermine did not differ statistically significantly in the investigated parameters m, m_1 and m_2 due to induced systemic acquired resistance by this growth regulator. SRC of the other four groups differed statistically significantly against the control SRC in all parameters investigated.

The spectral distributions of the fluorescence differences between averaged spectra of control and treated five groups of leaves, calculated using equation (1), are presented in Figure 7. The differences are smallest for the case Spermine/Control.

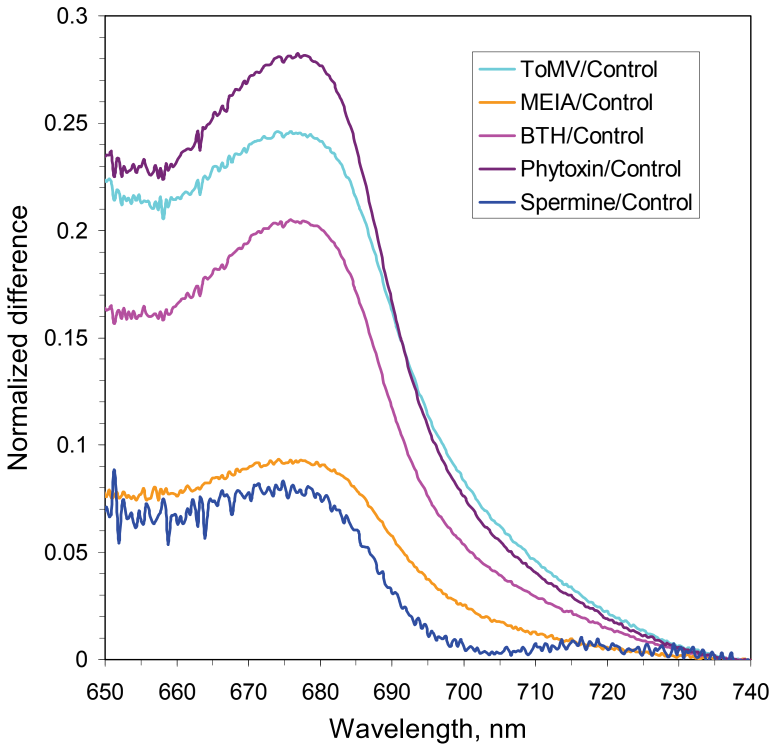

Figure 7. Normalized spectral distribution $\Delta F(\lambda)$ for data on the 14^{th} day after the infection

3. Conclusions

Statistically significant changes were established on the 7^{th} and 14^{th} day after inoculation of tomato plants by ToMV in the fluorescence spectra for all cases of plant treatment by the growth regulators MEIA, BTH, Phytoxin VS and Spermine in the spectral range 650-740 nm. The partial recover of the physiological status of plants was most clearly indicated by the spectra of Spermine treated plants on the 14^{th} day where the number of statistically significant differences was lower than on the 7^{th} day after treatment. The results from the leaf fluorescence analysis are in correlation with the spectral reflectance analysis and the serological (DAS-ELISA) results for presence of ToMV performed on the same plants. They prove the chlorophyll fluorescence technique as a promising tool for express and reliable diagnosis of viral infections.

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