Spectral remote sensing measurements for detection of viral infections in tobacco plants (*Nicotiana tabacum* L.)

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The ability to identify diseases in an early infection stage and to accurately quantify the severity of infection is crucial in plant disease assessment and management. A greenhouse study was conducted to assess changes in the leaf spectral reflectance and fluorescence of young tobacco plants during viral infections with the aim to evaluate the remote sensing spectral measurements as a tool for early identification and discrimination of different diseases. At the growth stage of 2-4 expanded leaf, the plants were inoculated with tobacco sap from leaves with clearly manifested infection symptoms of the four most widely spread in Bulgaria viruses: Cucumber mosaic virus - CMV, Tomato spotted wilt virus - TSWV, Tomato mosaic virus - ToMV and Potato virus Y - PVY. The leaf reflectance and fluorescence spectra were collected on the 7th day after inoculation using an Ocean Optics USB2000 spectrometer in the spectral ranges 450-850 nm and 600-850 nm, respectively. Specific differences in reflectance spectra between virus-infected and uninfected tobacco leaves were observed in four wavelength intervals: green (520-580 nm), red (640-680 nm), red edge (690-710 nm) and near infrared (720-760 nm). Results of statistical analysis by applying Student's t-criterion showed statistically significant differences against reflectance data of uninfected leaves at least in three of the investigated spectral ranges in dependence on the virus specificity. The fluorescence spectra of all infected leaves differed statistically significant in the spectral range 640-680 nm. The results of spectral analysis qualify the two remote sensing techniques as a promising tool for cost-effective, non-destructive method for early detection of viral infections.

Introduction

The plants or plant populations become stressed when biotic or abiotic factors adversely affect their growth and development. Stress or disease can be expressed in various ways. Some of the symptoms may include changes in plant morphology such as leaf curling, wilting or stunting, and chlorosis, necrosis or abscission of plant parts [1]. Such symptoms can be observed but it may be difficult to see early symptoms and quantify them accurately, precisely and rapidly. However, remote sensing methods provide means whereby such changes in plants can be detected and assessed. In general, the detection of plant stress or diseases by remote sensing is based on the assumption that adverse environmental factors interfere with photosynthesis or the physical structure of the plant, affecting the absorption of light energy and leading to an alteration in the reflectance spectrum and chlorophyll fluorescence of the plant [2].

It has been suggested that plant spectral properties at the visible (VIS) and near-infrared (NIR) ranges can assist in the development of specific signatures for a specific stress in a given plant species. Studies have been conducted with various crops to distinguish diseased leaves from healthy leaves [3, 4]. Virus diseases of plants are especially damaging and lead to a heavy reduction of the yield and quality of the production. Many viral infections are known to cause changes in leaf pigments, biochemical components and metabolic alterations in infected leaves [5]. These pathological conditions of plants can influence the spectral characteristics of leaves that can be detectable in the VIS and/or the NIR ranges of the electromagnetic spectrum. Thus, the differences between spectral reflectance of healthy and infected leaves can be used to identify the health status of a plant. By comparing leaf reflectance measurements in the VIS and NIR ranges, a reduction in chlorophyll was detected in the early stages of disease development in *Nicotiana debneyi* plants infected with Tomato mosaic virus, even though visible symptoms were not apparent until several days later [6]. Sasaki et al. [7] distinguished diseased cucumber leaves from healthy leaves at an early infection stage on the basis of the leaf spectral reflectance in the 500, 600 and 650 nm wavebands. Carter and Knapp [8] showed the importance of the chlorophyll concentration on the spectral signature of leaves. The spectral reflectance around 700 nm was found to be highly correlated with total leaf chlorophyll content. The optical response in the 700 nm waveband to stress, as well as corresponding changes in reflectance that occurred in the green-yellow range of the spectrum, were explained by the general tendency of stress to reduce leaf chlorophyll concentration. In some cases a dark green colour of the leaves was observed in result of the viral infection. In these cases the photosynthetic energy is greater than that of the plants which are not infected [9].

Even more information about the stress responses of a leaf may be obtained from the fluorescence emission. A small percent of ultraviolet and VIS 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 physiological status of the plant can be probed by means of chlorophyll fluorescence sensing, allowing the distinguishing of normal from stressed condition in intact plant material [10]. Daley [11] studied the effects of Tobacco mosaic virus on tobacco leaves fluorescence, finding high intensity spots in correspondence of the regions where the infection occurred. Peterson and Aylor [12] found a similar pattern on bean leaves infected by bean rust, stressing that significant changes in chlorophyll fluorescence preceded visual symptoms by 3-5 days. Scholes and Rolfe [13] considered the effects of crown rust on fluorescence of oat leaves, finding again a higher emission by infected regions 3-4 days before the appearance of chlorotic lesions.

The most widely spread viruses in Bulgaria are: Cucumber mosaic virus - CMV, Tomato spotted wilt virus - TSWV, Tomato mosaic virus - ToMV and Potato virus Y - PVY. The most widespread among them is CMV often reaching epidemiological spreading and when this occurs in times which are characterized by friendly climate conditions for developing of aphids, serious economical losses occur. The second place is occupied by TSWV. This virus is transmitted by thrips tabaci. CMV and TSWV have a wide set of hosts: tomatoes, pepper, cucumbers, tobacco, decorative plants and weeds. The worldwide distributed PVY is currently regarded as one of the main problems in seed potato production. PVY attacks also tomatoes, tobacco, pepper, many weeds. In nature it is transmitted by aphids [9, 14]. ToMV usually affects tomatoes, pepper, cucumbers, tobacco and weeds. In contrast to the rest viruses, ToMV is transmitted by sap or contact with inoculated plants, soil, seeds and many species of aphids.

The aim of this study is to evaluate the leaf spectral reflectance and chlorophyll fluorescence measurements as a tool for early identification and discrimination of different virus diseases in the plants. A greenhouse study was conducted with young tobacco plants inoculated with four different viruses. These diseases lead to different physiological effects which reduced the content of photosynthetic pigments.

Materials and methods

Plant material and treatment

Greenhouse studies were conducted at the Plant Protection Institute, Kostinbrod, Bulgaria, with young tobacco plants *(Nicotiana tabacum L.),* cultivar Nevrocop 1146 during the growing period May-June 2008. At stage of 2-4 expanded leaves, the plants were inoculated with infected tobacco sap with clearly manifested disease symptoms of ToMV, CMV, TSWV and PVY. The uniformity of inoculum was performed in 0.1 M sodium-potassium phosphate buffer pH 8, containing 2% sodium sulphite and ascorbic acid in ratio 1:1(w\v).

After extracting, the sap obtained was used for inoculation of test tobacco plants. We used the following virus isolates: PVY- potato isolate of origin Samokov town, for TSWV - isolate 4ZA-94 from Zanthedeshia of origin Negovan town, CMV Uo(S-I) widely spread in Bulgaria and originating from field cucumbers in Suhindol town, ToMV tomato isolate from Zimniza town. The inoculum of ToMV was diluted with distilled water to 1:50. PVY, CMV and TSWV homogeneities were diluted 1:5 with the buffer. After shading during 24 hours, the inoculated plants were transferred in a growth chamber at 25°C, illumination 3500-4000 luxes and photoperiod 16/8 hours day and night. After 7 days, the inoculated leaves were used for analysis. As control plants, healthy tobacco plants treated only with buffer were used.

Spectral remote sensing methods

I. Leaf spectral reflectance

Leaf spectral reflectance was measured using an Ocean Optics spectrometer USB2000 in reflectance mode [15]. The data were collected in the VIS and NIR ranges (450-850 nm) at a spectral resolution (halfwidth) of 1.5 nm in 1170 spectral wavebands. The measurements were carried out using an experimental setup in laboratory. The light signal from the object studied is conducted to the entrance lens of the spectrometer by a fibre-optic cable directed perpendicular to

the measured surface. The light source is Narva spectral lamp, type T-R1, providing homogeneous illumination of the leaf surfaces. In the beginning of each measurement the emitted spectrum of the source was registered from a diffuse reflectance standard WS1 (white plastic). The single measurement area (pixel) was with a diameter of 1 cm. The control of the spectrometer and the acquisition and processing of data are carried out by means of specialized software.

II. Chlorophyll fluorescence

The spectral measurements of the chlorophyll fluorescence were carried out in laboratory using a spectrometer USB2000 in the spectral range 600-850 nm where the main part of the emitted from leaves fluorescence radiation is concentrated. The spectra were obtained in 850 wavelengths at a step of 0.3 nm. As a source of actinic light, a light diode LED 05B470Y-10C 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 600-850 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 were dark adapted for 10 min before the measurements of fluorescence spectra. The abaxial side of the leaves was irradiated with actinic light and the exited fluorescence was measured from the adbaxial leaf surface.

Statistical methods

The Student's t-criterion was applied for analysis of the statistically significant differences between the means of parameters derived from the reflectance and fluorescence spectra of uninfected and virus-infected leaves. The analysis of reflectance data was performed in 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 four investigated spectral intervals: green (520-580 nm), red (640-680), red edge (690-710 nm) and near infrared (720-760 nm) in which changes in the spectral reflectance characteristics (SRC) were observed. The fluorescence spectra were analyzed in the wavelength λ = 685 nm at which the maximum of relative differences was observed for all the cases studied.

Results and Discussion

Fig. 1 shows photos of the investigated tobacco leaves: a) untreated and infected with viruses: b) CMV; c) TOWV; d) ToMV and e) PVY. Visible injures of the leaves were not observed because of the early stage of development of the viral infections (a week). Very light blends in the leaf colour depth appeared in the infected leaves in comparison with the control as well as in the leaf smoothness and thickness.

The averaged SRC over all measured areas of the uninfected and infected leaves are shown in Fig. 2. It is seen that the values of SRC of infected with PVY and ToMV differ significantly in the green, red and NIR spectral ranges. In the red edge a small shift (2-4 nm) of these two SRC to the lower wavelengths was observed.

SRC of the infected by TSWV and CMV tobacco leaves practically do not differ in the green spectral range from those of the uninfected leaves. In the red and NIR ranges, SRC of the infected and control leaves differ significantly. In the red edge, the SRC of infected leaves are shifted to the longer
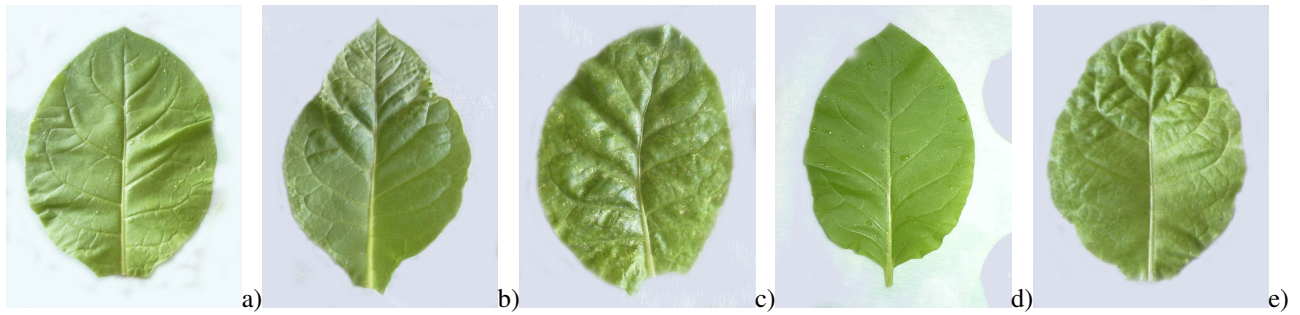
Fig. 1. Photos of investigated tobacco leave: a) uninfected and infected with b) CMV; c) TOWV; d) ToMV and e) PVY viruses
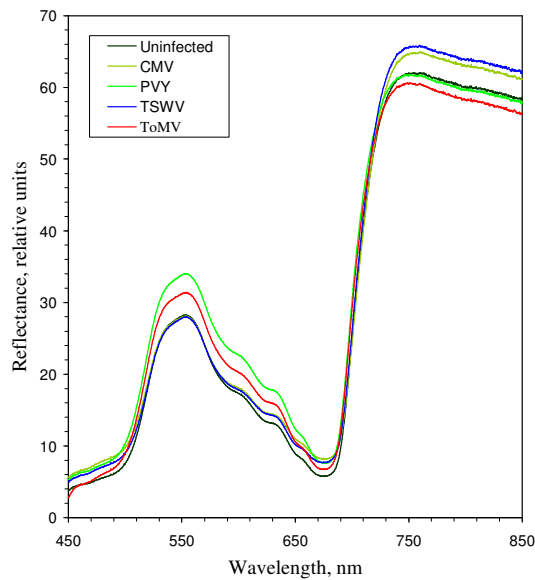

Fig. 2. Averaged spectral reflectance characteristics of uninfected and infected tobacco leaves

wavelengths.

The results of application of the Student's t-criterion are set out in Table I (PVY and ToMV) and Table II (CMV and TSWV). For the sake of simplicity, the notation λ_i / λ_{ic} stands for the ith wavelength ($i = 1,...,8$) in which pairs of SRC means of control and treated (separately by PMV, ToMV, CMV, TSWV) leaves are compared by t-criterion.

TABLE I
Significance p-level of the t-criterion in the cases of PVY and ToMV infection

Viral infection	PVY		ToMV		Control
Pairs compared	p_{st}	mean	p_{st}	mean	mean
λ_1 / λ_{1c}	<0.001	26.62	<0.001	24.64	21.24
λ_2 / λ_{2c}	<0.001	32.65	<0.001	30.20	26.98
λ_3 / λ_{3c}	<0.001	33.96	0.002	31.34	28.24
λ_4 / λ_{4c}	<0.001	8.34	0.003	7.25	6.15
λ_5 / λ_{5c}	<0.001	36.36	<0.001	34.99	31.16
λ_6 / λ_{6c}	<0.001	53.68	0.150	52.23	51.12
λ_7 / λ_{7c}	0.005	56.77	0.590	55.43	55.03
λ_8 / λ_{8c}	0.793	61.59	0.092	60.41	61.76

TABLE II
Significance p-level of the t-criterion in the cases of CMV and TSWV infection

Viral infection	CMV		TSWV	
Pairs compared	p_{st}	mean	p_{st}	mean
λ_1 / λ_{1c}	0.296	21.73	0.982	21.26
λ_2 / λ_{2c}	0.927	26.93	0.653	26.72
λ_3 / λ_{3c}	0.725	28.06	0.588	27.93
λ_4 / λ_{4c}	<0.001	8.55	<0.001	8.00
λ_5 / λ_{5c}	<0.001	30.28	0.0012	32.18
λ_6 / λ_{6c}	0.227	51.38	<0.001	53.25
λ_7 / λ_{7c}	0.005	56.05	<0.001	57.63
λ_8 / λ_{8c}	0.006	64.81	<0.001	65.66

Table I shows that statistically non-significant differences ($p>0.05$), were obtained between SRC of control and infected leaves only in NIR spectral range. For SRC of infected by CMV and TSWV leaves, Table II, non-significant differences were observed in the green range.

It is worth noting that for the cases with $p<0.05$ the value of ratio R = mean SRC $_{infected leaves}$ / mean SRC $_{control leaves}$ was observed within the interval 1-1.4. The largest R values in average were found for PVY, while the smallest values were observed for CMV. These results were obtained using the data for means shown in Table I and Table II. The ratio R may be regarded as indicator of the degree of influence of viral infections over the SRC of tobacco leaves.

The course of fluorescence spectra measured over 19 areas of uninfected leaves is shown in Fig. 3. All spectra are normalized against the values of the second maximum of the curves at $\lambda = 737$ nm. The doted curve shows the relative change of the fluorescence emission. Fig.4 shows the averaged over all measurements normalized fluorescence spectra of uninfected and infected tobacco leaves. It is seen that PVY and ToMV cause largest changes against the control spectrum. Fig. 5 illustrates the spectral ranges of relative changes of the fluorescence emission of uninfected and infected leaves. The five distributions are Gaussian shaped with a maximum at $\lambda = 685$ nm for all of them. The values of these curves were calculated as averaged sums of all possible positive differences between pairs C of normalized areas

belonging to data set n of control spectra. Their number may be calculated using the well-known formula:

$$C_n^{(2)} = \binom{n}{2} = \frac{n!}{2!\,(n-2)!}, \qquad (1)$$

where: C is the number of combinations of two elements and n is the number of measurements.
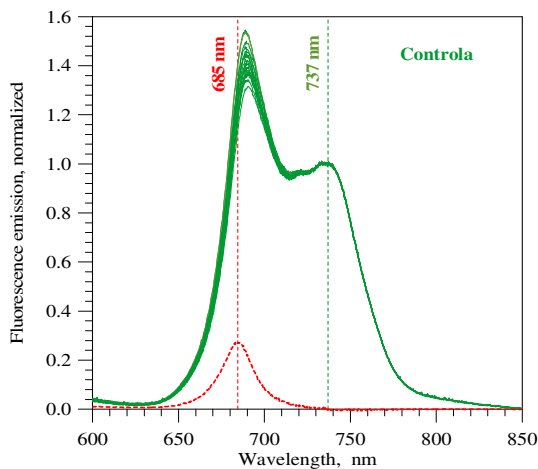

Fig. 3. Normalized fluorescence spectra of uninfected leaves and relative difference
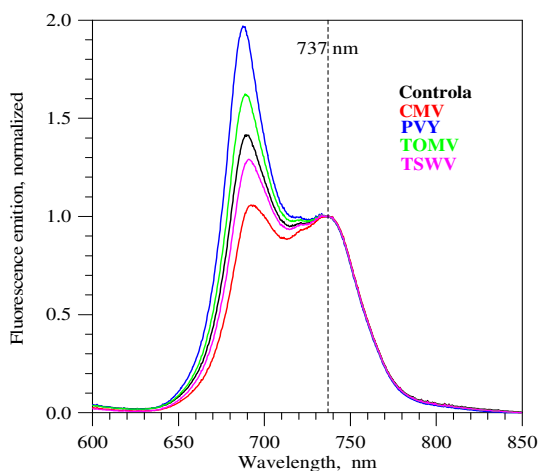

Fig. 4. Normalized fluorescence spectra of uninfected and infected tobacco leaves
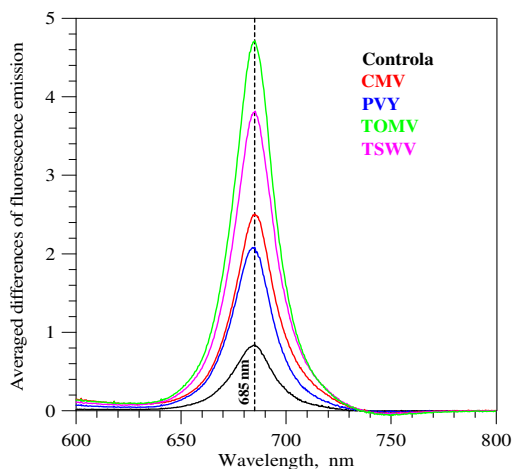

Fig. 5. Relative changes of the fluorescence emission of uninfected and infected tobacco leaves

The results after applying of t-criterion (Table III) indicate that in all of the cases of infection the means of the leaf fluorescence differed statistically significantly at $p<0.05$.

TABLE III
Significance p-level of the t-criterion for the differences between the normalized fluorescence spectra

Experiment	Control	CMV	PVY	ToMV	TSWV
Number of spectra	19	20	19	19	20
Means	1.292	0.868	1.896	1.510	1.124
p_{st} at $\lambda = 685$ nm		<0.001	<0.001	0.038	0.035

Conclusions

Using leaf spectral reflectance and chlorophyll fluorescence measurements we analyzed the influence of CMV, TSWV, ToMV and PVY infections of young tobacco plants growth in greenhouse. We found that the virus PVY is most injurious for tobacco plants in comparison with the rest three herein studied. Thus, by combining information from two remote sensing techniques, each of which detects a different basic physiological response, the sensitivity at diagnosing and quantifying different biotic stresses is greatly improved. The leaf spectral reflectance and fluorescence spectra proved to be promising tools for cost-effective, non-destructive methods for early detection of viral infections in tobacco plants.

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