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GROUND BASED HYPERSPECTRAL REMOTE SENSING FOR DISEASE DETECTION OF TOBACCO PLANTS

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Abstract

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Greenhouse experiment was conducted in the Institute "N. Poushkarov", Department Plant Protection Kostinbrod, with young tobacco plants infected with TSWV (Tomato Spotted Wilt Virus). Remote sensing technique, spectral reflectance, was applied for detecting and assessing the development of the viral infection. At growth stage 4-6 expanded leaf some of the plants were inoculated with TSWV by using infected material from a pepper fruit with severe symptoms of yellow spotting. Hyperspectral reflectance data of healthy (control) and infected leaves was collected by a portable fibre-optics spectrometer in the visible and near-infrared spectral ranges. The measurements were conducted on the 14th and 20th days after the inoculation. Spectral reflectance analyses were performed in green, red, red edge, and NIR regions. The differences between the reflectance spectra of control and infected leaves were assessed by means of the Student's t-criterion at ten selected wavelengths and first derivative analysis. The viral concentration in the leaves was determined by the serological method DAS-ELISA. On the 14th day no visual changes in some of the infected leaves occurred but the differences of averaged reflectance spectra against the control were statistically significant at four of the investigated wavelengths and the presence of TSWV was established, i.e. the latent infection has been occurred. Reflectance spectra of the other leaves differed statistically significantly at eight wavelengths. On the 20th day the statistical analysis indicates an increase of the number of statistically significant differences and the shift of the red edge position, i.e. the infection is deepening that is in agreement with serological analyses.

Key words: hyperspectral reflectance data, TSWV, tobacco plants, DAS-ELISA

Abbreviations: TSWV - Tomato Spotted Wilt Virus, VIS - visible, NIR - near-infrared, DAS-ELISA - Double Antibody Sandwich Enzyme-linked Immunosorbent Assay, SWIR - short wave infrared region, SRC - spectral reflectance characteristics, PCR - polymerase chain reaction, OD - optical density

Introduction

Monitoring and plant health assessment have an important role in controlling diseases in agricultural crops which can result in yield loss, poor quality and economic loss (Everett et al., 1999). The bacterial, fungal, and viral infections, along with infestations by insects result in plant diseases and damage. Upon infection, a plant develops symptoms that appear on different parts of the plants causing a significant agronomic impact (López et al., 2003). The contemporary practices for struggle with the plant disease infestations mainly consist of indiscriminately spraying with agrochemicals over the field which is an expensive and time consuming process (Apan et al., 2005; Sankarana et al., 2010). Here is to note that conventional sprayings with pesticides are in limited use in the control of viral diseases owing to the peculiarities of viral pathogens. To minimize economic loss and to reduce environmental pollution, it is necessary accurately to assess the disease of the crops so that the effective control measures to be applied timely to the infected plants. Until recently, damage evaluation of diseases has been largely done by visual inspections (Moshou et al., 2004). But with the advancement of the technology in agriculture sector, the remote sensing techniques have been used to monitor disease epidemics in ag-

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ricultural crops (Moshou et al., 2005; Delalieux et al., 2007; Yang et al., 2009).

Remote sensing applications in agriculture began over the past 25 years with sensors for soil organic matter, and have quickly diversified to include satellite, aerial, and hand held or tractor mounted sensors (Mulla, 2013). Many of the earlier studies focused on broad spectral bands such as the visible (VIS) and near-infrared (NIR) spectral ranges (350 nm to 1300 nm), which could be used in vegetation studies (Carter, 1993; Panuelas and Filella, 1998). With the development of hyperspectral remote sensing technologies, researchers have benefited from significant improvements in the spectral and spatial properties of the data, allowing for more detailed plant and environmental studies (West et al., 2003; Lee et al., 2010). Today, electromagnetic wavelengths in use are ranging from the ultraviolet to microwave portions of the spectrum (350 nm to 2500 nm). The data obtained are in many hundreds of contiguous spectral bands, and this allows improved analysis of specific compounds, molecular interactions, crop stress, and biophysical or biochemical characteristics related to plant status (Muhammed, 2005; Naidu et al., 2009; Krezhova et al., 2012). Moreover, the high spatial and spectral resolution of hyperspectral technology increases the potential to detect anomalies in the normal plant production processes at an early stage, before the damage occurs (Panda et al., 2010; Lee et al., 2010). This possibility has lately driven most researches in the field of vegetation stress and disease detection (Bravo et al., 2003; Delalieux et al., 2007; Krezhova et al., 2005).

Vegetation behavior depends on the nature of the vegetation itself, its interactions with solar radiation and other climate factors, and the availability of chemical nutrients and water within the host medium, usually soil or water in marine environments. In recent years, there has been an expanding body of literature concerning the relationship between the spectral reflectance properties of vegetation and the structural characteristics of vegetation and pigment concentration in leaves (Sims and Gamon, 2002; Panda et al., 2010). The spectral characteristics of vegetation are governed primarily by scattering and absorption characteristics of the leaf internal structure and biochemical constituents, such as pigments, water, nitrogen, cellulose and lignin (Datt, 1998; Coops et al., 2002; Nielsen and Simonsen, 2011). Foliar pigments are the main determinants controlling the spectral responses of leaves in the VIS part of the spectrum (Zarco-Tejada et al., 2000; Coops et al., 2003). Figure 1 (Sanderson, 2007) shows a typical reflectance curve of green vegetation and main factors controlling leaf reflectance. Reflected radiation is low in VIS due to the strong absorption of chlorophylls and carotenoids. In particular, leaf chlorophyll content is a major factor that dictates the amount of reflected energy and is directly associated with photosynthetic capacity and productivity (Gaussman, 1977; Curran et al., 1992). In the NIR and short wave infrared (SWIR) regions the reflectance is influenced by the amount of standing biomass and cellular structure, whereas leaf and canopy water content is responsible for the absorption wells that characterize the SWIR (Jensen, 2007; Panda et al., 2010).

Vegetation stress is a result of complex physiological processes. Stress symptoms show up as photosynthesis decline. With the persistence of the stress (i.e. pollution, water deficiency, high temperature), stress induced mechanisms become dominant and give rise to acute or chronic injury (damage phase), depending on the stress tolerance threshold (Ustin et al., 2004). The plant response to stress implies biochemical and morphological changes during this phase that is therefore irreversible. In stressed vegetation, leaf chlorophyll content decreases, thereby changing the proportion of light-absorbing pigments, leading to a reduction in the overall absorption of light (Murtha, 1982; Zarco-Tejada et al., 2000). These changes affect the spectral behavior of plants through a reduction in green reflection and an increase in red and blue reflections, resulting in changes in the normal spectral reflectance patterns of plants (Zarco-Tejada et al., 2000; Krezhova, 2011). More recent work has highlighted the importance of more specific narrow-band regions such as the red edge (maximum slope of vegetation reflectance from 680 nm to 720 nm) for predicting plant stress (Pu et al., 2003; Clay et al., 2006; Campbell et al., 2007).

Tomato spotted wilt virus is one of the most wide-spread and damaging viruses affecting vegetable crops. Tospoviruses similar or identical to TSWV are recognized as infecting more than 1000 monocot and dicot species worldwide (Peters, 1998). Some show symptoms and some do not. TSWV

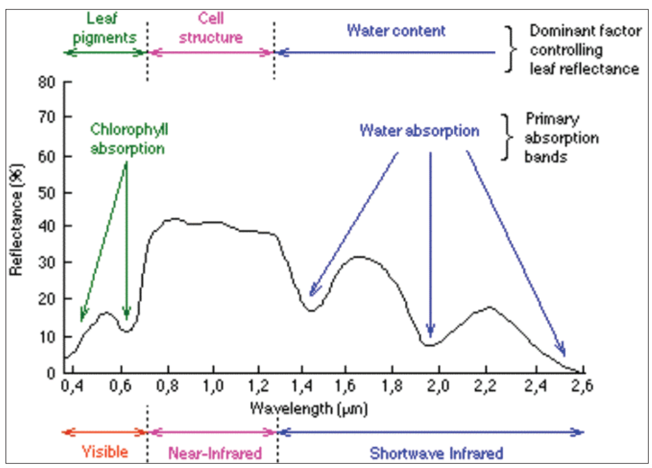

Fig. 1. Dominant factors controlling leaf reflectance

causes significant damage to solanaceous vegetables such as tomatoes, potatoes and peppers, but also to lettuce and a wide range of herbs and ornamental, most often dahlias and chrysanthemums. Cucumber infections are symptomless. Plant resistance and various strategies that reduce viral transmission from plant to plant are the only effective methods of controlling tospoviruses (Roberts and Pernezny, 2003; Momol et al., 2005).

In the present paper the advanced remote sensing technique, hyperspectral reflectance, was applied for detection of viral infection at young tobacco plants infected with TSWV. The possibility of using hyperspectral data as a tool for early or pre-visual detection of the disease and for discriminating the degree of the leaf infection was investigated. The early detection of plant diseases (before the onset of disease symptoms) could be a valuable source of information for executing proper management strategies to prevent the development and the spread of diseases.

Materials and Methods

Plant material and inoculation

As a model system we used young tobacco plants (*Nicotiana tabacum* L.), cultivar Samsun NN, grown in a greenhouse under controlled conditions (22-25°C, humidity 75-85%, photoperiod of 16/8 h, light intensity 3000–4000 lux). At growth stage 4-6 expanded leaf some of the plants were inoculated with TSWV. Infected material from diseased pepper fruit with severe symptoms of yellow spotting was used. One gram of infected pepper tissue was homogenized in 2 ml 4°C 0.1M Potassium-sodium buffer, pH 7.0, with 0.2% Na_2SO_3 and 0.2% Ascorbic acid. A part of the plants was not inoculated (control).

TSWV causes a wide variety of symptoms including wilting, stem death, stunting, yellowing, poor flowering, and sunken spots, etches, or ring spots on leaves. These spots later turn brown, followed by a general browning of leaves that die and appear drooped on stems. Plant are often stunted, and with the droopy leaves, give one the impression that they are wilted. Green fruit show concentric rings of yellow or brown alternating with the background green color, and striking brown rings occur on red-ripe fruit (Momol et al., 2004). One of the investigated tobacco leaves on the 20th day after the inoculation is shown in Figure 2.

Hyperspectral reflectance

Recent developments in technologies in agricultural sector lead to a demand for non-destructive methods of plant disease detection. It is desirable that the plant disease detection tool should be rapid, specific to a particular disease, and sensitive for detection at the early onset of the symptoms (Lòpez et al., 2003). The spectrometric techniques are unique disease monitoring methods that are used to detect diseases and stress caused by various factors in plants.

Remote sensing method, hyperspectral reflectance, was applied to monitoring and plant disease detection and assessment. Terrestrial materials reflect and absorb the light differently at the wavelengths. Therefore, it is possible to differentiate among materials (vegetation, soils, rocks, etc.) by their spectral signatures, the measured reflected electromagnetic radiation at varying wavelengths. Green vegetation species all have unique spectral features, mainly because of the chlorophylls and carotenoids, and other pigments and water content. The spectral reflectance is a function described by the dependence of the ratios of the intensity of reflected light to the illuminated light on wavelengths in VIS (400-700 nm), NIR (700-1200 nm), and SWIR (1200-2500 nm) spectral ranges, (Figure 1). Chlorophyll strongly absorbs radiation in the red and blue regions but reflects in the green range. The internal structure of healthy leaves acts as excellent diffuse reflector in NIR. Measuring and monitoring the NIR reflectance is one way that scientists can determine how healthy (or unhealthy) vegetation may be.

Acquisition of data

Hyperspectral reflectance data were collected from fresh detached leaves by a portable fibre-optic spectrometer USB2000 (Ocean Optics, USA) in the VIS and NIR spectral ranges (450-850 nm) at a spectral resolution (halfwidth) of 1.5 nm and step band 0.3 nm in 1170 spectral wavebands. The measurements were carried out using an experimental setup in laboratory. The light source is a halogen lamp providing

Fig. 2. TSWV symptoms on tobacco leaf on the 20th day after the inoculation

homogeneous illumination of the leaf surface. The spectral reflectance characteristics (SRC) of the investigated plants were determined as the ratio between the reflected from the leaves radiation and those one reflected from the diffuse reflectance standard. Specialized software was used for data acquisition and processing.

DAS-ELISA testing

In last decade molecular techniques of plant disease detection have been well established. The sensitivity of the molecular techniques refers to the minimum amount of microorganism that can be detected in the sample. The commonly used techniques for disease detection are ELISA (Enzyme-linked immunosorbent assay) and PCR **(polymerase chain reaction),** PCR and real-time PCR. In the ELISA-based disease detection (Clark and Adams, 1977), the microbial protein (antigen) associated with a plant disease is injected into an animal that produces antibodies against the antigen. These antibodies are extracted from the animal's body and used for antigen detection with a fluorescence dye and enzymes.

To identify the presence or absence of TSWV within the leaf tissue of tobacco plants, cultivar Samsun NN, DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay) testing was performed. For the analysis a commercial kit (LOEWE Biochemica GmbH, Sauerlach, Germany) with polyclonal IgG, specific for TSWV were used. After the substrate reaction was allowed to proceed for 30 min at room temperature, absorbance values were determined by a spectrophotometer SUMAL PE (Karl Zeiss, Jena, Germany) at a wavelength of 405 nm (A_{405}). Samples that gave DAS-ELISA values of greater than two and half times the mean of control (healthy plants) were considered to be positive or virus carriers. The A_{405} values were corrected by subtracting the mean of the buffer control absorbance values from sample values. The positive control was infected with TSWV indicator plants (pepper fruit with symptoms of chlorotic concentric ring spots). The absorbance values from all ELISA tests were plotted on histograms.

Data analysis

Ten bands at key wavelengths were selected to relate physiological status of the plants (chlorophyll content, cell structure, water content) to leaf reflectance based on our investigations of plant stress during the last years (Krezhova and Kirova, 2011; Krezhova, 2011). The bands were located in four spectral ranges: green (520-580 nm, maximal reflectivity of green vegetation), red (640-680nm, maximal chlorophyll absorption), red edge (680-720 nm, maximal slope of the reflectance spectra) and NIR (720-770 nm), where changes appeared between the reflectance spectra of healthy and unhealthy plants. The middle of the bands is at wavelengths: λ_1 = 475.22 nm, λ_2= 489.37 nm, λ_3 = 524.29 nm, λ_4 = 539.65 nm, λ_5 = 552.82 nm, λ_6 = 667.33 nm, λ_7 = 703.56 nm, λ_8 = 719.31 nm, λ_9 = 724.31 nm, and λ_{10} = 758.39 nm. Statistical (Student's t-criterion and cluster analysis) and derivative analysis were applied to the data. T-criterion was applied for determination of the statistical significance of differences ($p<0.05$) between the means of sets of the values of the reflectance spectra of un-inoculated and inoculated leaves on the 14th and 20th days after the inoculation. Statistical analysis was performed using STATISTICA 7.1 (StatSoft, 2005). First derivative analysis was applied in order to assess the position of the inflection points of the averaged SRC in the red edge region which is very informative for presence of damage in the plants. Tobacco leaves were derived into three groups based on the degree of injury.

Hierarchical cluster analysis on the groups of spectral data was performed. This is the major statistical method for finding relatively homogeneous clusters of cases based on measured characteristics. It starts with each case as a separate cluster, i.e. there are as many clusters as cases, and then combines the clusters sequentially, reducing the number of clusters at each step until only one cluster is left. The clustering method uses the dissimilarities or distances between objects when forming the clusters.

Results and Discussion

From all inoculated plants three groups of leaves were selected for spectral measurements with the aim to analyze the sensitivity of hyperspectral reflectance for revelation of disease and early diagnosis of symptoms in plants at different stages of infections. In first (symptomless) group no visual symptoms were observed in the leaves. In some places the leaf tissue is thinner than the control. The leaves from the other two groups were with symptoms. Some of the leaves were with cuticle thickness and chlorotic yellow ring or concentric spots (with symptoms) and a part of leaves were with dark brown spots (necrotic spots).

Averaged SRC over all measured areas (up to 20 for each leaves group) of the control and infected tobacco plants, taken on the 14th day after the inoculation, are shown in Figure 3. Differences in reflectance spectra were established at a great number of wavelengths in all investigated spectral ranges. The values of SRC of the two groups of leaves with symptoms are higher in the green, red, and NIR spectral ranges against the control. In the red edge, a shift of the SRC values to the shorter wavelengths was observed. For the rest (symptomless) group SRC is slightly decreased in green and red regions (520-680 nm). In NIR region values of SRC are higher than the control.

The results from the statistical analysis by applying the Student's t-criterion on the data sets of all groups investigated tobacco leaves on the 14th day after the inoculation are displayed in Table 1. SRCs of symptomless leaves differed statistically significant against the control in four wavelengths in green region. The SRCs of the other two groups differed statistically significantly in all investigated wavelengths except in λ_{10} for group with necrotic spots. The differences between SRCs of all leaves and control SRCs are non-significantly at three wavelengths in NIR region.

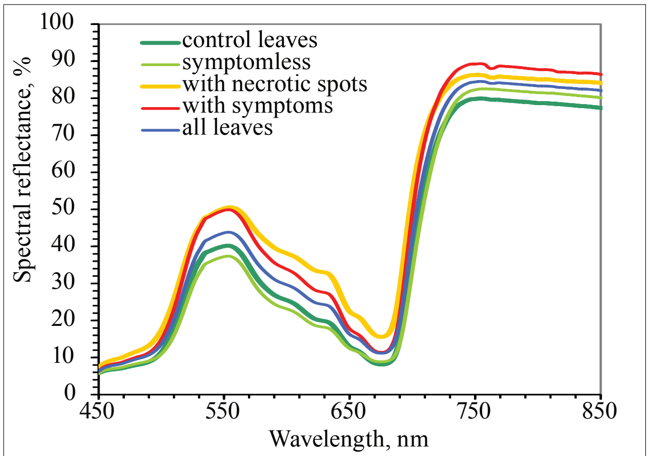

Fig. 3. Averaged SRC of tobacco leaves on the 14th day after the inoculation with TSWV

First derivative analysis was applied to the averaged SRC of the control and three groups of infected tobacco leaves. Figure 4 shows the maximums of the derivatives of SRC of all investigated groups. For the two groups with symptoms the maximums are shifted to the lower wavelengths (about 3-5 nm) whereas for the symptomless group – no shift is observed. Figure 5 shows the maximum of the derivative of SRC of control leaves, which is located at 697.17 nm, and the maximum of the derivative of SRC of all leaves infected with TSWV that occurs at 694.81 nm. This maximum is shifted to the blue spectral region which is an indicator for the presence of the disease in tobacco plants.

The results from the hierarchical cluster analysis applied to a set of data measured on the 14th day after the inoculations of tobacco plants are displayed in Figure 6. A hierarchical tree diagram is made to show the linkage points. The clusters are linked at increasing levels of dissimilarity. According to the Tree cluster analysis, the spectral data was separated in three groups. In the first group, nearest to the control were symptomless leaves. Next group, statistically different from the control's one, contained the data of leaves with symptoms and the SRC of all leaves. The leaves with necrotic spots belong to a separate group, statistically different of both the other groups and on the long distance of the healthy plants. These results are in accordance with the results from spectral analysis and t-criterion.

The results from serological analysis by DAS-ELISA test for the leaf samples from tobacco plants, cv. Samsun NN, taken on the 14th day after inoculation with TSWV are

Table 1
Tobacco plants, cv. Samsun NN: p-values of the Student's t-criterion for TSWV infection on the 14th day after the inoculation

Pairs compared	Control mean	P	TSWV Symptomless mean	P	TSWV Nectotic mean	P	TSWV Symptoms	P	TSWV All leaves mean
λ1/λ1c	7.64	+	8.01	+++	11	+++	9.53	++	9.24
λ2/λ2c	8.77	ns	8.94	+++	13.11	+++	11.19	+++	11.01
λ3/λ3c	31.73	+++	26.5	+++	41.07	+++	39.62	+++	36.63
λ4/λ4c	38.93	+++	33.57	+++	48.62	+++	47.95	+++	44.32
λ5/λ5c	40.21	+++	34.9	+++	50.52	+++	49.56	+++	45.83
λ6/λ6c	8.92	ns	9.32	+++	16.95	+++	12.49	+++	12.55
λ7/λ7c	44.79	ns	38.58	+++	61.38	+++	56.79	++	52.16
λ8/λ8c	67.77	ns	64.82	+++	78.53	+++	78.07	ns	72.42
Λ9/λ9c	72.1	ns	70.49	++	81.45	+++	81.9	ns	76.02
Λ10/λ10c	79.86	ns	81.83	ns	86.07	+++	88.12	ns	82.14

Statistical significance at: ns - not statistical significance of the differences, + - $P<0.05$, ++ - $P<0.01$, +++ - $P<0.001$

shown on the diagram in Figure 7. All samples showed positive extinction values (optical density, OD), higher than the value of cut off (0.088), i.e. presence of viruses was confirmed. For the leaves of the symptomless group the extinction value is also positive, i.e. the latent infection in some of the leaves has been occurred. Symptomless (latent) infections are very important for spreading out the virus diseases. The plants with latent infections are virus carriers, but not virus diseased.

The averaged SRC over all measured areas (up to 20 for each group of plants) of the control and infected tobacco plants, taken on the 20th day after the inoculation, are shown in Figure 8. It is seen that the values of SRC of the two groups of leaves with symptoms are increased in all spectral range against the control. In the red edge, a shift of the SRC values of infected leaves to the shorter wavelengths was observed. For the rest symptomless group, the SRC is decreased in green and NIR region because the leaves in some places were thinner, i.e. latent infection was present.

The results from the statistical analysis by the Student's t-criterion on the data sets of all investigated tobacco leaves on 20th days after the inoculation specify an increase of the number of statistically significant differences between spectral reflectance of control and infected plants as well as a shift of the red edge position to the lowest wavelengths (up to 4 nm) which is an indicator that the infection is going deep. For symptomless group SRCs differed statistical-

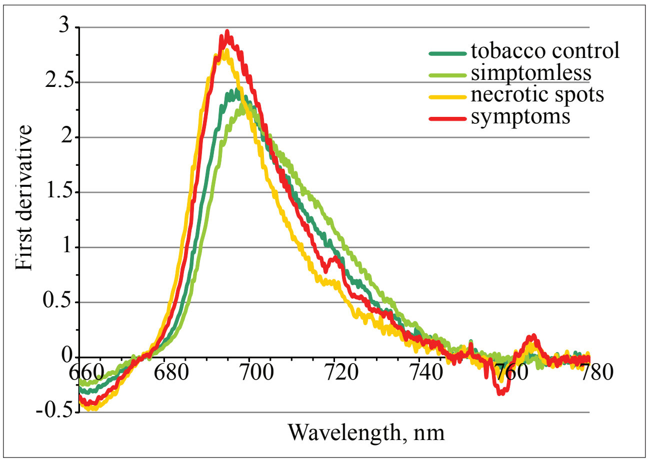

Fig. 4. Maximum of the first derivatives on SRC of control and infected with TSWV tobacco leaves on the 14th day after the inoculation

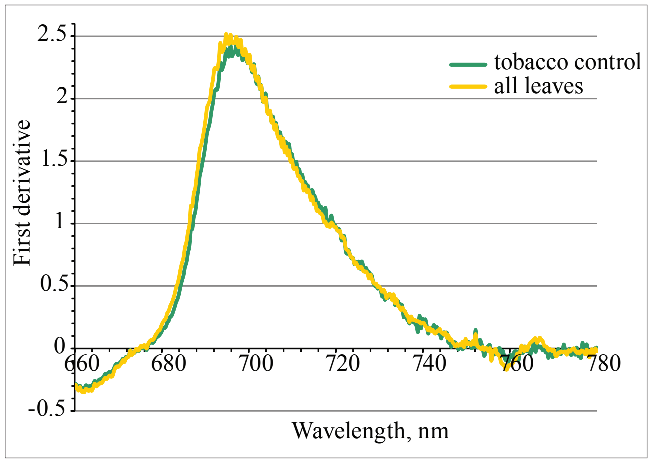

Fig. 5. Maximum of the first derivatives on SRC of control and all infected with TSWV tobacco leaves on the 14th day after the inoculation

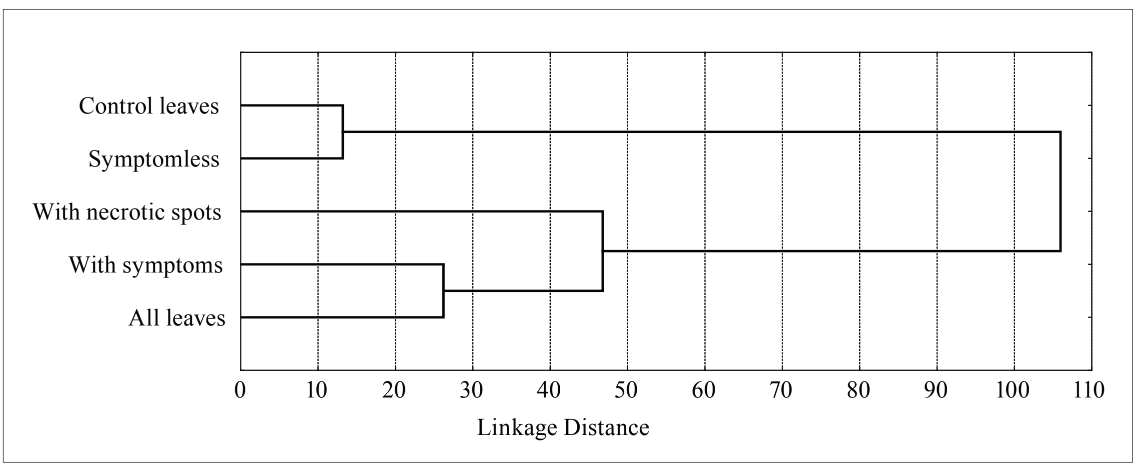

Fig. 6. A hierarchical tree diagram of data set of tobacco plants measured on the 14th day after the inoculation

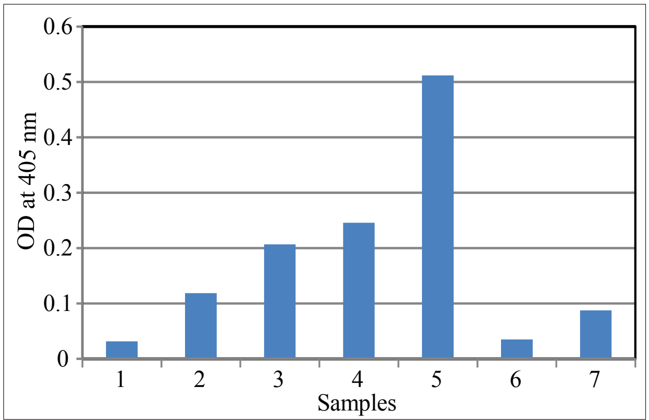

Fig. 7. DAS-ELISA of leaf samples from tobacco plants, cv. Samsun NN, taken on the 14th day after inoculation with TSWV

Legend: 1 - control (negative) – leaf from healthy tobacco plant; 2 – symptomless plants; 3 – with symptoms; 4 – with necrotic spots; 5 – positive control from pepper fruit, infected with TSWV with symptoms of chlorotic concentric ring spots; 6 – buffer for extraction of leaf samples; 7 – cut off (borderline for distinguishing ELISA positive and negative for virus samples).

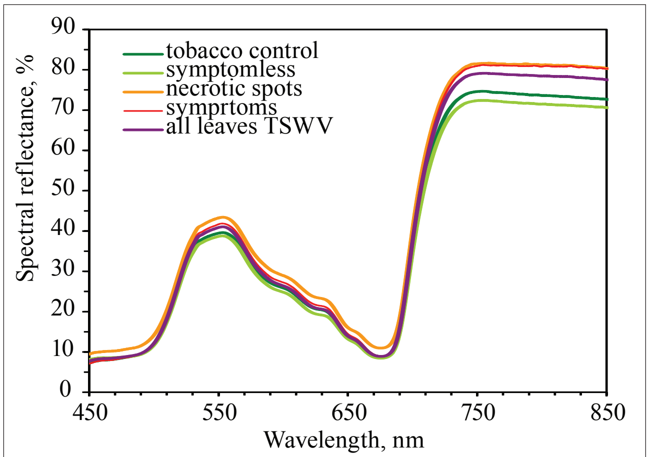

Fig. 8. Averaged SRC of tobacco leaves on the 20th day after the inoculation with TSWV

ly significant at seven of investigated wavelengths. SRCs of all leaves and group with symptoms differed non-significant in the blue region at two wavelengths. SRC of group with necrotic spots differed significant at all wavelengths.

The first derivative analysis was applied to the SRC of control and three groups of infected tobacco leaves in order to assess the position of the inflection points in the red edge

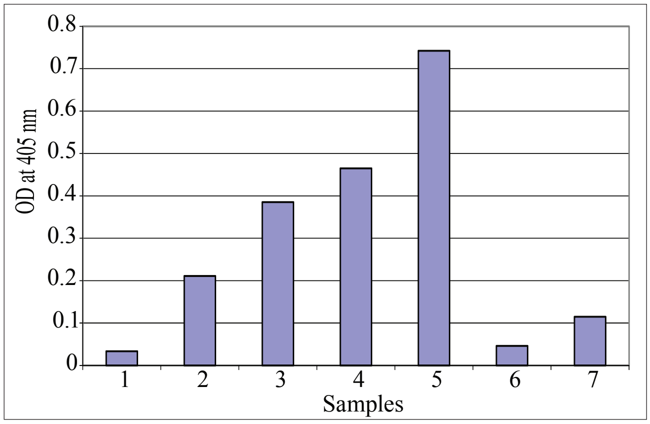

Fig. 9. DAS-ELISA of leaf samples from tobacco plants, cv. Samsun NN, taken on the 20th day after inoculation with TSWV

Legend: 1 - control plants; 2 – symptomless plants; 3 – with symptoms; 4 – with necrotic spots; 5 – positive control from pepper fruit; 6 – buffer; 7 – cut off

region. The maximum of the derivative of SRC of control leaves is located at 697.81 nm while for the group of all leaves infected with TSWV it occurs at 693.49 nm. It is shifted to the blue spectral region and the shift is bigger than on the 14th day after the inoculation which is an indicator for the development of the disease.

The results from serological analysis by DAS-ELISA test are illustrated in Figure 9. They show the increase of the quantity of TSWV in all leaf groups which is an indicator that the infection is deepening.

Conclusions

The impact of viral infections on young tobacco plants, cultivar Samsun NN, caused by the widely spread in Bulgaria Tomato spotted wilt virus (TSWV) on the leaf spectral reflectance was investigated. The sets of hyperspectral reflectance data were analyzed by means of statistical (Student's t-criterion, cluster analysis) and derivative (first derivative) analyses. For assessment of the presence and the degree of the viral infections serological analyses via DAS-ELISA techniques were applied on samples from the same leaves. The results of all applied techniques were subjected to comparative analysis. The strong relationship established between the results from the remote sensing study and the serological analyses provides evidence for the importance of remote sensing hyperspectral reflectance data for conducting, easily and without damage, rapid assessments of plant health condition.

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