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Spectral Remote Sensing of the Responses of Soybean Plants to Environmental Stresses

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1. Introduction

Precision agriculture, site-specific application of inputs tailored to the needs of the crop, is one of the new ways that modern agriculture could potentially maintain or enhance crop yields and minimize environmental pollution. Knowledge about variations in vegetation species and community distribution patterns, alterations in vegetation phenological cycles, and modifications in the plant physiology and morphology provide valuable insight into the climatic, edaphic, geologic, and geophysical characteristics of Earth's areas (Janetos & Justice, 2000). During the past decade remote sensing techniques have been widely used to monitor crops throughout their growing period to help in making decisions for good agricultural practices. Spectral remote sensing methods provide the possibility for early, efficient, objective, and non-destructive evaluation of plant responses to different stress factors of the environment (Campbell et al., 2007; Govender et al., 2009; Li et al., 2010). Field remote sensing applications addressed agriculture and forestry survey, fire detection and fire-fuel mapping, mineral mapping, and atmospheric modelling. Airborne, space-borne and hand-held technologies are commonly used to investigate the spectral responses of plants. Hyperspectral remote sensing makes possible to enhance significantly the spectral measurement capabilities over conventional remote sensing sensor systems, as well as to improve the spectral information content. This entails detailed assessment of the changes in the physiological stage of plants in response to the changes in the environment (Zarco-Tejada et al., 2002; Steele et al., 2008a), detecting of early-stage vegetation stress (Krezhova et al., 2005; Ouyang et al., 2007), discriminating land cover types (Flamenco-Sandoval et al., 2007), leaf pigment concentrations (Coops et al., 2003), modelling quantitative biophysical and yield characteristics of agricultural crops (Delalieux et al., 2009a; Chatzistathisa et al., 2011).

Ground-truth is essential for detecting plant stress, and two commonly used ground-based optical methods, leaf spectral reflectance and chlorophyll fluorescence, are reviewed for their usefulness and practical application. When these methods were combined with remarkable advances in Global Positioning System (GPS) receivers, geographic information systems (GIS), and enhanced crop simulation models, remote sensing technology has the potential to transform the ways that growers manage their lands and implement precision farming techniques (Upchurch, 2003; Hatfield, et al., 2008; Shuanggen & Komjathy, 2010). To obtain accurate and complementary comparative assessments for plant responses to the environmental changes, methods have been applied from different research fields - remote

sensing, plant physiology, biochemistry, virology, etc. Early detection of stress could identify plant physiological condition at larger spatial and temporal scales before visible effects are apparent (Krezhova et al., 2009a; Chatzistathisa et al., 2011).

Soybean *(Glycine max L.)* is one of the most important and valuable agricultural species of legume, as its high protein content is of primary importance for human food and animal feed. Soybean is the leading oilseed crop produced and consumed worldwide. Fat-free soybean meal is a primary, low-cost, source of protein for animal feeds and most pre-packaged meals, as well as a good source of protein for the human diet. The soy vegetable oil is another valuable product of processing the soybean crop and a biofuel feedstock (FAOSTAT, 2011).

Soybean yields have steadily increased in the past 30 years owing to a combination of genetic and management improvement. Rapid soybean demand increases in the last decade challenge the reliability of supply, stock levels, and reasonable pricing. In order to meet the demand, there are two alternatives: increase planted hectares or increase yield. Increasing soybean hectares by substituting for other crops (e.g. sunflower in Argentina or cotton in the United States), utilizing pasture (e.g. Santa Fe, Argentina or Mato Grosso, Brazil) or replacing native vegetation (e.g. cerrado in Brazil) has been the most expedient manner to increase soybean output (Masuda & Goldsmith, 2009). Going forward available farmland for soybean production will be limited by decreasing quantities of land not already in production, increased farmland loss for urbanization, heightened sensitivities about agricultural uses of land, and weak property rights in regions such as Africa that constrains the employment of modern agricultural methods (Goldsmith, 2008). Although soybean use for biodiesel production may require expansion of land area devoted to soybean in some parts of the world, such an expansion is not likely in Europe and North America. Hence, yield increases will become the major source for sustaining further increases in soybean production, particularly in these two significant regions of the world. The design of soil and crop management strategies that fully exploit the climatic and genetic yield potential of soybean remains a key challenge to achieve this goal (USDA, 2009).

Gene transformation and genetic engineering are likely to be of assistance in increasing crop yields worldwide, particularly in less-developed areas affected by low crop productivity and malnutrition. Crop transformations restricting the influence of biological pests could contribute to increased crop productivity (Miflin, 2000). Once pests are controlled, either using genetically improved plants or various management options, the further step could be to increase the inherent yielding capability of plants. Yield potential may be increased by improving of the overall physiological capacity of plants and by preventing the negative consequences of abiotic stresses. Increasing leaf photosynthetic rates appear to be a straightforward way of increasing crop yields. Considerable physiological research has been carried out to select and breed for genotypes with superior photosynthetic rates, and was successful in identifying such cultivars in maize, wheat and soybean (Masclaux et al., 2001; Habash et al., 2001; Sinclair et al., 2000). In soybean, the trait is inherited quantitatively (Sall & Sinclair, 1991).

To achieve high yield potential, soybean must sustain high photosynthesis rates and accumulate large amounts of nitrogen (N) in seeds. It exists in leaves primarily as ribulose biphosphate carboxylase/oxygenase and there is generally a strong relationship between N per unit leaf area and photosynthesis (Sinclair, 2004). Biological nitrogen fixation (BNF) and mineral soil or nitrogenous fertilizers are the main sources of meeting the N requirement of high-yielding soybeans. However, antagonism between nitrate concentration in the soil

solution and the nitrogen fixation process in the nodules is the main constraint the crop faces in terms of increasing N uptake when no other abiotic stress that reduces BNF activity occurs. A number of reviews have been published on BNF in legumes (Unkovich & Pate, 2000; Hardarson & Atkins, 2003) and soybean in particular (Hungria et al., 2005, Hungria et al., 2006). However, these summaries were mostly qualitative and did not emphasize the role of BNF and inherent soil fertility in high-yielding soybean systems. Likewise, many studies evaluating the response of soybean to N fertilization show conflicting results that make it difficult to draw a general conclusion about soybean response to N fertilizer (Ray et al., 2005; Osborne & Riedell, 2011).

In sustained agronomic systems, both the BNF and an adequate management of the organic matter, play important roles. However, the BNF importance as a source of N for agriculture has diminished in recent decades as increasing amounts of fertilizer N have been used for the production of food and cash crop. Currently, it's of great practical importance because the use of nitrogenous fertilizers has resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates) and eutrophication of lakes and rivers (Barker & Sawyer, 2005; Salvagiotti et al., 2008). Thus, legumes are also essential to improve the soil fertility and quality of agricultural lands and to reclaim eroded or barren areas, making them crucial for agricultural and environmental sustainability (Saikia & Jain, 2007). However, legume BNF in crop species is very sensitive to environmental constraints such as salinity, drought, and light in particular (Ibanez et al., 2008; Salehi et al., 2008; USDA, 2009).

Many fundamental studies are dedicated on how plants detect and respond to stresses in their environment. The stress factors cause changes in the normal physiological processes of all cultural and wild plants. They influence the metabolism, photosynthesis and enzyme activity, and lead to a dramatic reduction of yields and to deterioration of the output quality. The physiological condition of plants is indicative of plant productivity and adaptability to stress and it is a general indication of the environment in which they grow (Alia et al., 2006; Gray et al., 2010). Research on biotic stresses includes the molecular mechanisms used by viruses, bacteria, fungi, and nematodes to incite disease and those used by plants to resist infection (Li et al., 2008; Yang et al., 2009; 2008; Delalieux et al., 2009b). Research on abiotic stresses includes molecular mechanisms by which plants resist such unfavourable conditions as drought, flooding, chilling, light, excess salts, toxic metals, and pollutants (Flawers, 2004; Jones, 2007; El-Nahry & Hammad, 2009).

Soil salinity is one of the widespread environmental factors and the major factor limiting plant production in many areas of the world. This is especially true in arid and semi-arid regions of the world like some regions of Bulgaria. Salinity influences almost every aspect of the physiology and biochemistry of plants (Arida & Das, 2005). High exogenous salt concentrations affect seed germination, water deficit, cause ion imbalance of the cellular ions resulting in ion toxicity and osmotic stress (Yousfi et al., 2007; Singha et al., 2010). As with most cultivated crops, the salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties and the stage of growth. One of the important impacts of salinity on plants is that it essentially creates a physiological drought in plants (Munns, 2002). The ability to monitor or evaluate the efficiency of cropping production systems in saline areas can be significantly improved by applying remote sensing techniques (Thenkabail et al., 2004; Campbell et al., 2007).

Light is one of the most important environmental factors regulating plant development and the expression of plant genes. A plant's ability to maximize its photosynthetic productivity depends on its capacity to sense, evaluate, and respond to light quality, quantity, and

direction. Stratospheric ozone depletion has led to elevated levels of ultraviolet-B (UV-B) radiation (280-320 nm) on the surface of the Earth. Increased UV-B levels have negative effects on human health (Norval et al., 2006) as well as on the plant development, morphology, and physiology (Jia Gio & Wang, 2008). Low influence UV-B radiation stimulates distinct responses, such as the accumulation of UV-absorbing pigments. Low influence of UV-B was also found to stimulate the transcript levels of a robust set of genes involved in stress responses (Rock, 2000). Although the effects of UV-B on plants are well characterized at the physiological level, little is known about the effects of UV-B on underground (root) physiology, particularly in interaction with other environmental factors. An increasing number of studies have been designed to test the interactions of environmental factors on plants, such as the interaction between UV-B and water stress (Cechin et al., 2008), interaction between salinity and Fe deficiency (Zancan et al., 2006), and interaction between UV-B radiation and Fe deficiency (Zancan et al., 2008).

The aim of this chapter is to show some aspects of the recent applications of non-destructive remote sensing techniques, hyperspectral leaf reflectance and chlorophyll fluorescence, for detection and discrimination of the effects of some environmental stresses (salinity and enhanced UV-B radiation) on young soybean plants, as well as the influence of the biological nitrogen fixation on the spectral responses of the plants to stress. To evaluate the effects of a given stress a comparative analysis was performed between the changes of the leaf spectral reflectance and fluorescence data and the stress markers such as phenols, malondialdehyde, thiol groups, proline and hydrogen peroxide, and chlorophyll content that were estimated by biochemical methods.

2. Remote sensing methods

Generally, remote sensing refers to the activities of recording, observing, and perceiving (sensing) objects or events at far away (remote) places. Remote sensing is defined as a science and technology by which the characteristics of objects or events of interest can be identified, measured or analyzed without direct contact with the sensors. The spectral information relies on the properties of the light after multiple interactions, i.e., reflections, transmissions, and absorptions with the object. The information needs a physical carrier to travel from the objects/events to the sensors through an intervening medium. The electromagnetic radiation which is reflected or emitted from an object is the usual source of remote sensing data. However any media such as gravity or magnetic fields can be utilized. Remote sensing is a technology to identify and understand the object or the environmental condition through the uniqueness of their spectral responses. This technology offers advantages such as viewing parts of the Earth at different scales (synoptic view), monitoring of regions that are very remote or with restricted access, ability to obtain imagery of an area of the Earth at regular intervals over many years and to evaluate changes in the landscape as well as capability to distinguish anthropogenic effects.

A basic assumption made in remote sensing is that specific targets (soils of differed types, water with varying degrees of impurities, rocks of differing lithologies, or vegetation of various species) have an individual and characteristic manner of interacting with incident radiation that is described by the spectral response of that target. Different materials reflect and absorb visible (VIS) and infrared light differently at different wavelengths. They have different colours and brightness when seen under the sun. Thus, the targets can be differentiated by their 'spectral reflectance signatures', a term used to describe the spectral

response of a target. The variety of earth's surface materials is enormous, and therefore the recording of their spectral signatures (also known as spectral library) requires substantial financial and time investments. With the development of hyperspectral technology, the spectral resolution of hyperspectral sensors have reached less than 10 nm, which is sufficient for creating a continuous spectral curve from 350-2500 nm to detect subtle changes in the spectral behaviour of the earth objects. For years, efforts have been made to establish such datasets and pool them for general use through spectral libraries. Such spectral libraries are maintained by many organizations including the Johns Hopkins University (JHU), the Jet Propulsion Laboratory (JPL), and the United States Geological Survey (USGS). Many of these datasets are made available with commercial remote sensing image processing software packages.

2.1 Spectral reflectance

Methods based on reflectance makes use of VIS, near infrared (NIR), and short-wave infrared (SWIR) sensors to form images of the earth's surface by detecting the solar radiation reflected from targets on the ground. These methods rely on making measurements simultaneously in one or more wavebands. Spectrophotometers offer the simplest solution for spectral reflectance measurements. They measure spectrum of light reflected from the whole (mostly circular) field of view of the instrument but not provide any spatial information on the pattern of reflection (West et al., 2003). Earlier studies utilized multispectral sensors with low spatial (60 m to 80 m) and spectral resolution commonly collected in four to seven spectral bands in the VIS and NIR regions. Spectral resolution refers to the number and width of the portions of the electromagnetic spectrum measured by the sensor. A sensor may be sensitive to a large portion of the electromagnetic spectrum but have poor spectral resolution if it captures a small number of wide bands. Spatial resolution defines the level of spatial detail depicted in an image and it is directly related to image pixel size. The spatial property of an image is a function of the design of the sensor in terms of its field of view and the altitude at which it operates above the surface (Smith, 2001a). Early airborne systems included a multispectral camera mounted on board a light aircraft. Spectrometers at this time were bulky, heavy instruments which were not easily transportable in the field and most measurements were taken in laboratories.

Remote sensing technologies have advanced significantly over the past 10 to 15 years. 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 (Thenkabail et al., 2004; Blackburn, 2007). These technologies acquire many hundreds of spectral bands across the VIS, NIR, and mid-infrared portions of the electromagnetic spectrum from 350 nm to 2500 nm, using satellite, airborne or hand-held devices. Advances in spectrometry and information technologies have resulted in state-of-the-art portable field instruments which allow for the collection of hand-held hyperspectral signatures. There are certain problems in the area of hyperspectral analysis connected with the optimal selection of bandwidth, number of bands and spatial as well as spectral resolutions and some constraints like data storage, communication bandwidth, discrimination/classification accuracy, minimum signal-to-noise ratio, sensor selection, data acquisition procedures and the cost factor.

The spectral reflectance responses are affected by factors such as soil nutrient status, the growth stage of the vegetation, the colour of the soil (which may be affected by recent weather conditions). In some instances, the nature of the interaction between incident

radiation and earth's surface materials will vary in time during the year, such as might be expected in the case of vegetation as it develops from the leafing stage, through growth to maturity and, finally to senescence. These responses also depend upon such factors as the orientation of the Sun, the height of the Sun in the sky (solar elevation angle), direction in which the sensor is pointing relative to nadir (the look angle), the topographic position of the target in terms of slope orientation, the state of health of vegetation if that is the target, and the state of the atmosphere.

Vegetation has a unique spectral signature which enables it to be distinguished readily from other types of land cover in an optical/infrared part of the electromagnetic spectrum. The spectral responses 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 (Sims & Gamon, 2002; West et al., 2003). 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 and pigment concentration in leaves (Gitelson et al., 2003; Blackburn, 2007; Sun et al., 2008; Hatfield et al., 2008). Chlorophyll pigment content is a major factor that dictates the amount of energy reflected or emitted and can be good indicator of crop health (Wu et al., 2008).

The function describing the dependence of the ratios of the intensity of reflected light to the illuminated light on wavelength in VIS (400-700 nm), NIR (700-1200 nm), and SWIR (1200-2400 nm) spectral ranges is the spectral reflectance characteristic (SRC) of the target. Fig.1 presents the typical spectral reflectance characteristics of green vegetation. The labelled arrows indicate the common wavelength bands used in optical remote sensing of vegetation: A - blue band, B - green band, C - red band, D - NIR band, and E - SWIR band. Reflectance is low in both the blue (450 nm) and red (670 nm) regions of the spectrum, due to absorption by chlorophyll for photosynthesis, also known as the chlorophyll absorption bands. It has a peak at the green region (550 nm) which gives rise to the green colour of vegetation. In the NIR region, the reflectance is much higher than that in the VIS band due to the cellular structure in the leaves. Hence, vegetation can be identified by the high NIR but generally low VIS reflectance. The reflectance of vegetation in the SWIR region is more varied, depending on the types of plants and the plant's water content. Water has strong absorption bands around 1.45, 1.95 and 2.50 μm. The SWIR band can be used in detecting plant drought stress and delineating burnt areas and fire-affected vegetation.
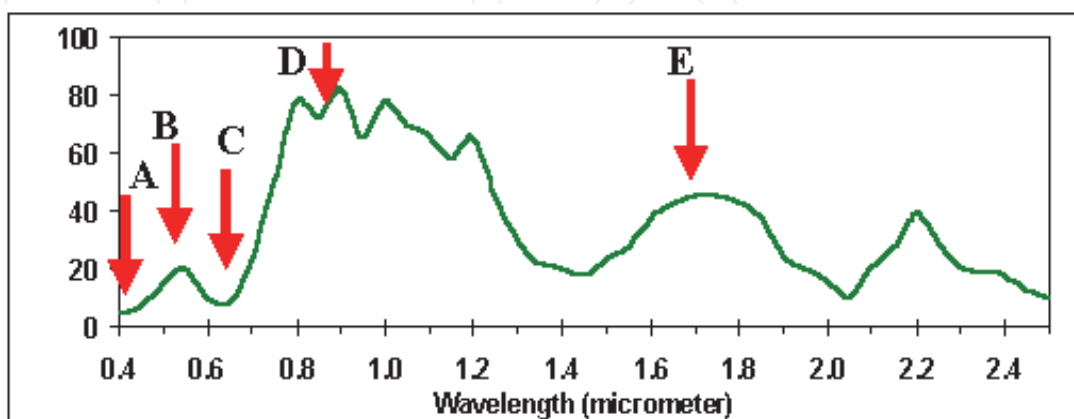

Fig. 1. Typical spectral reflectance characteristic of green vegetation in the VIS, NIR and SWIR ranges.

The shape of the reflectance spectrum is used for identification of vegetation type. For the same vegetation type, the reflectance spectrum also depends on other factors such as the leaf moisture content and health of the plants. Fig. 2 shows typical reflectance spectra of some species of green vegetation compared to a spectral signature for senescent leaves (Smith, 2001b).
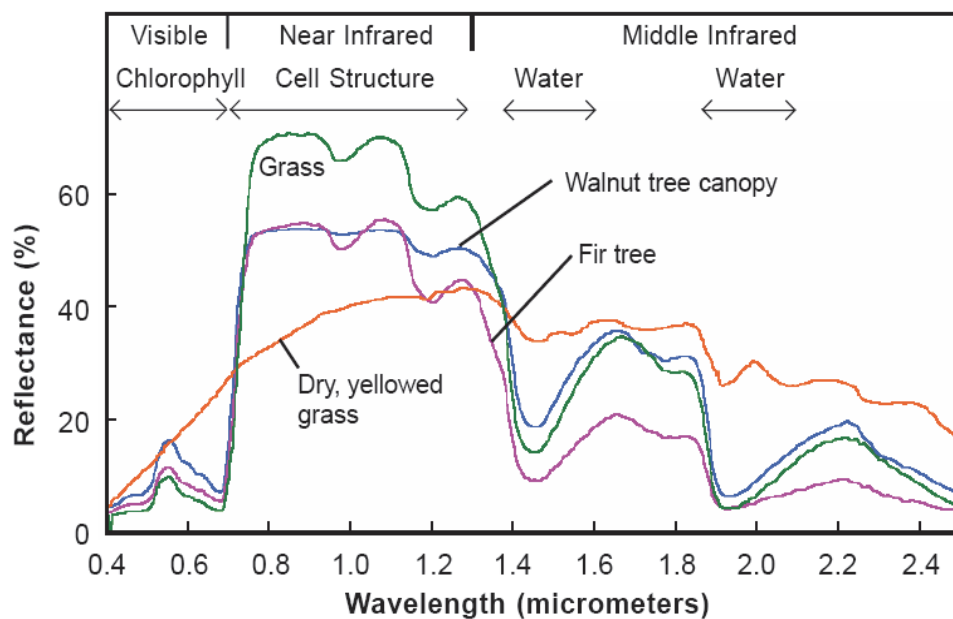

Fig. 2. Leaf reflectance spectra of different vegetation types.

In past decade, the research efforts were focused on the elucidation of some aspects of the link between the spectral responses and the physiology of plants under stress. 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 (Zarco-Tejada et al., 2000; Clay et al., 2006; Gang et al., 2010). These changes affect the spectral reflectance signatures 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; Campbell et al., 2007).

More recent works have 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 (Fitzgerald et al., 2006; Blackburn, 2007; Steele et al., 2008). The reflectance around red edge is sensitive to wide range of crop chlorophyll content and leaf internal scattering (Dawson & Curran, 1998). Experimental and theoretical studies show that red edge position shifts according to changes of chlorophyll content, N content, biomass and hydro status, age, plant health levels, and seasonal patterns (Filella & Penuelas, 1994; Pu et al., 2003; Hatfield et al., 2008;). These observations on red edge position can effectively be used to classify and distinguish different vegetation types and ages in the study.

Mathematical functions of two or more spectral bands are used rather than direct reflectance data to minimize the negative impact of interfering factors, such as the surrounding land cover, bare soil, or climatic/atmospheric conditions (McDonald et al., 1998; Huete et al., 2002). These functions are called vegetation indices (VIs), each designed for optimal correlation with a particular vegetation feature. The capacity of vegetation indices to

characterize natural canopies and agricultural crops has been demonstrated in numerous studies aimed at seasonal phenology (Carter, 1998; Qi et al., 2000), biomass prediction (Broge and Leblanc, 2001; Haboudane et al., 2004), mapping chlorophyll content (Haboudane et al., 2002). Numerous studies have documented the use of vegetation indices such as ratio vegetation index (RVI) and normalized difference vegetation index (NDVI) in the detection of crop stress (Kobayashi et al., 2001; Vigier et al., 2004; Yang et al., 2009). Combining individual spectral reflectance bands as simple ratio vegetation indices (SRVI) has been a common approach in remote sensing because it generally reduces the effects of spectral noise and allows for better temporal comparisons due to minimization of atmospheric effects (Carter & Miller, 1994). Commonly, SRVIs have consisted of the ratio of blue to red wavebands in an effort to detect responses due to changes in chlorophyll a and b concentrations. Gitelson et al. (2003, 2006) suggested the use of empirical vegetation indices, calculated from the reflectance of three wavelengths that were highly correlated with chlorophyll (Chl), carotenoid, and anthocyanin concentrations to estimate the content of foliar pigments in single leaves. Furthermore, various statistical and artificial intelligence methods have been used to analyze the remotely sensed data in agricultural crops. Among many, popular approaches include cluster analysis (Holden & LeDrew, 1998), principal component analysis (Zhang et al., 2002; 2003), partial-least square regression (Huang & Apan, 2006), artificial neural networks (Liu et al., 2008).

With the advent of hyperspectral remote sensing technology, more detailed data are potentially available. Therefore the extracting meaningful relationships of the overwhelming quantity of data are necessary. Currently, a variety of techniques have been used including a number of different vegetation indices, band absorption analysis, spectral mixture analysis, "red edge" position, statistical analysis, wavelet transform and neural networks (Thenkabail et al., 2004; Delalieux et al., 2007; Steele et al., 2008b).

2.2 Chlorophyll fluorescence

In recent years, chlorophyll fluorescence (ChlF) analysis has become one of the widely used techniques available to plant physiologists and has participated increasingly in plant ecology and physiology studies (Rolando & Little, 2003; Chaerle et al., 2004; Gielen et al., 2006). This analysis has been used more extensively to provide considerable information on the organization and function of the photosynthetic apparatus (Campbell et al., 2007). The chlorophyll molecule has the ability to absorb light energy and transfer it into the photosynthetic apparatus. Excess energy can be dissipated as heat or re-emitted as light at longer wavelength, i.e. chlorophyll fluorescence. The increase in efficiency of one of these three processes (absorption, fluorescence and thermal emission) will result in a decrease in yield of the other two. As such, the relative intensities of ChlF are strongly related to the efficiency of photochemistry and heat dissipation (Papageorgiou & Govindjee, 2004; Lichtenthaler et al., 2007; Delalieux et al. 2009b) and may provide additional data to detect plant stress in an early stage. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest.

Chlorophyll a (Chl a) is contributing largely to plant fluorescence emission. Excitation energy for this fluorescence is delivered from accessory antenna chlorophylls (Chl a and Chl b), absorbing light of blue and red wavelengths, and from carotenoids, absorbing photons of blue wavelengths. At room temperature, Chl a emits fluorescence in the red and NIR spectral regions between 650–800 nm, in two broad bands with peaks at λ_{max1} (684–695 nm) and λ_{max2} (730–740 nm) (Franck et al., 2002). The shorter wavelength emission is attributed to Chl a

mostly associated to PSII (Dekker et al., 1995), whereas the longer wavelength emission originates from antenna chlorophyll of both PSI and PSII (Agati et al., 2000; Buschmann, 2007). Several environmental factors, including water, salinity, light and nutrients, affect the process of photosynthesis and may lead to plant stress. Changes in chlorophyll function take place before changes in chlorophyll content, before any physical signs of tissue or chlorophyll deterioration are manifested in the plant, and therefore alterations in the fluorescence signal occur before any visible signs are apparent (Cambpell et al., 2007; Li et al., 2010). Under conditions of stress, some plant mechanisms for disposing of excess energy do not work efficiently, thus causing changes in the competing reactions of photochemistry, heat loss and fluorescence. Although the total amount of chlorophyll fluorescence is very small (only 2 or 3% of total light absorbed), measurement is quite easy. The spectrum of fluorescence is different to that of absorbed light with the peek of fluorescence emission being at longer wavelength than that of absorption. Therefore, fluorescence yield can be quantified by exposing a leaf to light of defined wavelength and measuring the amount of light re-emitted at longer wavelengths (Maxwell & Johnson, 2000).

Various fluorescence intensity ratios, combining the emissions at blue (F440), green (F520), red (F690), and NIR (F740) wavelengths, were proposed for probing the vegetation vitality status and stress responses (Buschmann et al., 2000; Mishra & Gopal, 2008). The red ChlF emission between 684-695 nm is strongly reabsorbed by the Chl pigments in the upper layer leaf cells (Agati et al., 1993; Dau, 1994), while the NIR ChlF between 730–740 nm is re-absorbed to a much smaller extent. Consequently, the ratio between the red and far-red ChlF bands (e.g. F690/F740) decreases with increasing leaf Chl content in a curvilinear relationship, which can be used as a good inverse indicator of Chl content changes due to plant growth or stress events (Buschmann, 2007). Finally, the UV excited blue-to-red/NIR fluorescence intensity ratios (F440/F690 and F440/F740) were proposed as indicators of the leaf physiological development (Stober et al., 1994; Meyer et al., 2003), but also as marker of the nutrition availability and stress occurrence (Heisel et al., 1996).

The red and NIR fluorescence emissions by Chl a are highly dynamic, being modulated by photochemical and non-photochemical quenching. These dynamic phenomena yielded important insights into the molecular processes of photosynthesis that occur within time-scales ranging from femtoseconds to minutes depending on the power of an actively applied actinic light (Govindjee, 1995; Nedbal & Koblizek, 2006; Baker, 2008). Most widely used field observations are active, using devices exciting the photosynthetic machinery with a measuring light and recording the induced fluorescence. Introduction of the pulsed amplitude modulation (PAM) fluorometer allowed non-imaging outdoor measurements in broad daylight (Schreiber et al., 1986). Fluorescence imaging was introduced in the laboratory by Omasa et al. (1987) and modified for field surveys in the mid-1990s by Nedbal et al. (2000). The laser pulses of actinic light, which can be discriminated from static and panchromatic background light, are applied to elicit fluorescent transients when measuring fluorescence from a distance (Cecchi et al., 1994; Corp et al., 2006). The footprint of such a light detection and ranging (LIDAR) laser beam can be expanded from several centimetres up to metres to cover larger observation areas or to decrease the power of the excitation source (Saito et al., 2005). The first field laser-induced vegetation fluorescence was observed by Measures et al., (1973). Lately, an eye-safe outdoor laser-induced fluorescence transient (LIFT) fluorometer has been constructed. This device is able to measure the fluorescence parameters and non-photochemical quenching or electron transport rate from a distance of about 30-50 m (Ananyev et al., 2005; Kolber et al., 2005). A new generation active field fluorescence

instrument, developed by Raimondi et al. (2007), was successfully employed in summer 2007 during the joint CarboEurope, FLEX, and Sentinel-2 ESA mission campaign. These ground-based active fluorescence-sensing techniques can be used whenever temporal monitoring of fluorescence transients is required regardless of the appearance of cloud cover.

Lately, chlorophyll fluorescence techniques proved to be a non-intrusive, fast and reliable attractive tool in ecophysiological studies, and have extensively been used in assessing plant responses to environmental stress.

3. Materials and treatments

3.1 Plant material

The experiments were carried out on young soybean plants (*Glycine max L., cultivar Pavlikeni 101).* They were grown as water cultures in a growth chamber under controlled conditions (16/8 photoperiod day to night, photon flux density 90 μmol m^{-2} sec^{-1}, humidity 60-70%, and temperature 25 ± 1 °C). Soybean seeds were surface sterilized with 70% ethanol and washed afterwards several times in distilled water. Then, they were let to germinate on a damp filter paper at 24 °C for three days in dark. On the 4th day seeds were transferred in plastic vessels with half strength well aerated Helrigel nutrient solution (Helrigel, 1898) and were put to growth clamber.

3.2 Induction of stresses

The first part of experiments was carried out to investigate the influence of a single environmental stress factor (salinity) on the spectral properties and physiological state of soybean plants. Plants were grown in nutrient solution with constant nitrate levels at all development stages (1st to 4th trifoliate expanded leaves). They were divided into three groups. These one kept only in nutrient solution were used as control (untreated). At the growth stage of 2nd trifoliate expanded leave to the nutrient solutions of the other two groups was added NaCl at concentrations 40 mM and 80 mM. Some of the investigated leaves are shown in Fig. 3. They are from plants salinity treated for 14 days.
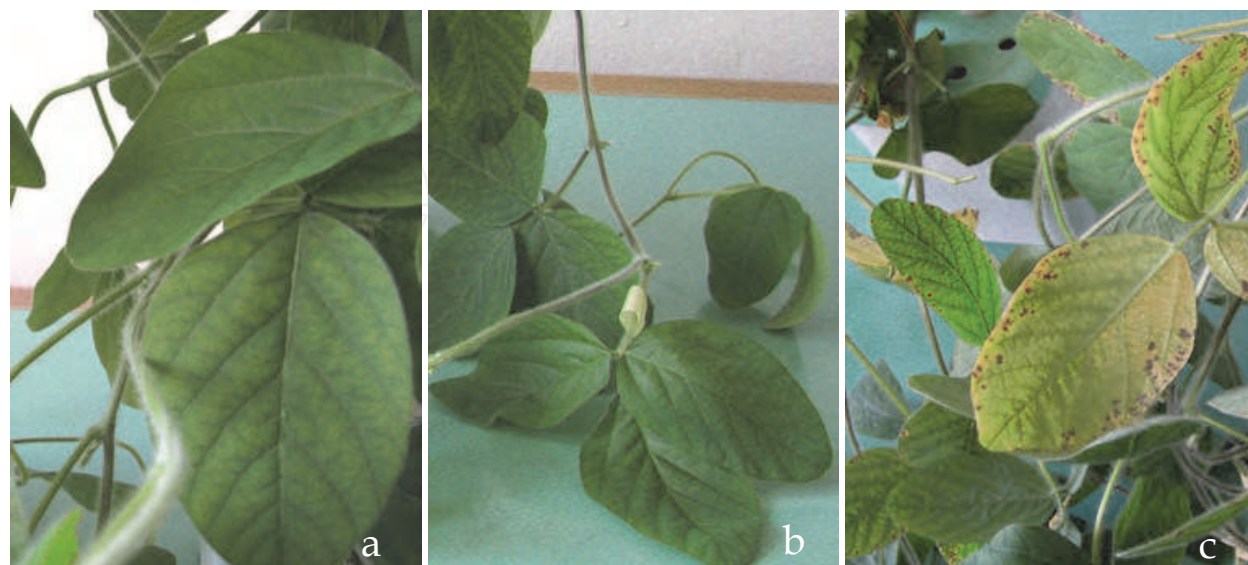

Fig. 3. Leaves from control (a) and treated with 40 mM NaCl (b) and 80 mM NaCl (c) soybean plants.

The second part of the experiments was focused on studying of the effect of the salinity on nitrogen fixing soybean plants. Three day's seedlings were inoculated with adding of 108 cells ml^{-1} suspension of Bradyrhizobium japonicum strain 273. After that they were transferred into plastic vessels with Helrigel nutrient solution. The nitrogen in the nutrient solution was equal to ¼ of the full dose until the growth stage of fully expanded 2nd trifoliate leaf, i.e. up to nodule forming and beginning of effective nitrogen fixation. The plants were salinity treated during the vegetative stage of growth from 2nd to 4th trifoliate expanded leaf for 14 days. Salinity was performed by means of adding in nutrient solution NaCl at two concentrations (40 mM and 80 mM). The control plants were kept only in nutrient solution. Figs. 4, 5, and 6 show the roots and nodules of nitrogen fixing control and treated with two NaCl concentrations plants on 14th day after the salinity.

The third part of experiments was aimed to investigate the effect of salinity stress and two consecutive stress factors (salinity and UV-B radiation) on young nitrogen fixing soybean plants. They were grown, nitrogen fixing, and salinity treated in the same way as the plants of the previous experiments.

Fig. 4. Control nitrogen fixing soybean plants: a) leaves; b) roots.

The investigated plants were divided into six groups. The first group consisted of untreated (control) plants. The second and third groups were only salinity treated by two NaCl concentrations. The plants of the fourth group were control (not salinized). The plants of fifth and sixth groups were treated for 14 days with 40 mM and 80 mM NaCl, respectively. Together with the control group they were irradiated with UV-B light for two hours. As a light source a lamp HPQ type with intensity 64.4 µmol m^{-2} s^{-1} was used at a distance of 25 cm. Fig. 7 a), b) shows some of investigated leaves from first (control for salinity treatment) and forth (control for treatment with UV-B radiation) groups. Fig. 8 a), b) and Fig. 9 a), b) show some leaves from plants treated with 40 mM NaCl and (40 mM NaCl + UV-B), and 80 mM NaCl and (80 mM NaCl + UV-B), respectively.

Fig. 5. Nitrogen fixing soybean plants treated with 40 mM NaCl: a) leaves; b) roots.

Fig. 6. Nitrogen fixing soybean plants treated with 80 mM NaCl: a) leaves; b) roots.
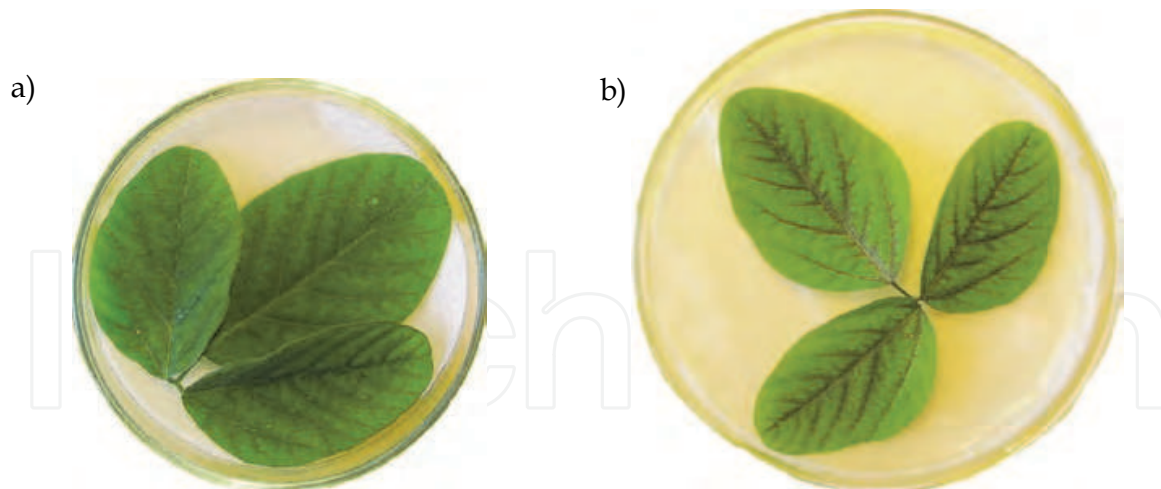
Fig. 7. Leaves from plant groups: a) first (control); b) fourth (control +UV-B).
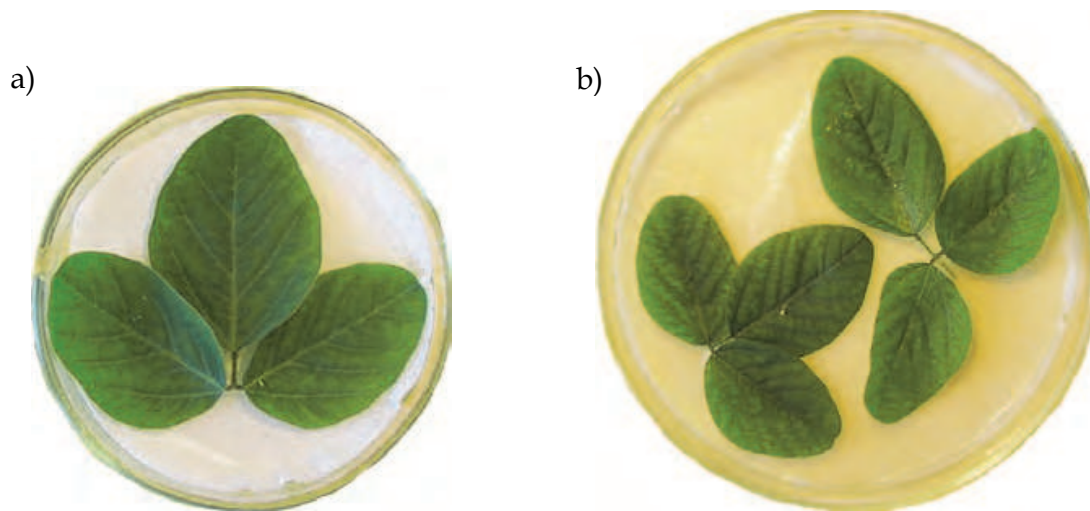

Fig. 8. Leaves from plant groups: a) second (40 mM NaCl salinity; b) fifth (40 mM NaCl + UV-B).

Fig. 9. Leaves from plant groups: a) third (80 mM NaCl salinity; b) sixth (80 mM NaCl + UV-B)

4. Biochemical methods

The biochemical parameters, stress markers such as phenols, proline, malondialdehyde (MDA), thiol groups, hydrogen peroxide (H_2O_2), glutathione, and leaf pigments (chlorophyll a and b) were determined to analyze the physiological stage of the plants and to perform a comparative analysis with the results from the spectral remote sensing methods. All stress markers were measured using a spectrophotometer Multiskan Spectrum (Thermo Electron Corporation). Fresh leaf materials (0.2 g) of soybean plants were homogenized at 4 °C in 0.1% cold trichloroacetic acid. The homogenates were centrifuged at 15000 g for 30 min. Then the supernatants obtained were used for determination of the stress markers after applying different methods.

Phenols and proline are important protective components of the plant cells and they accumulate when cells are in stress conditions. The antioxidant properties of phenols are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans et al., 1997; Khan et al., 2002). Plants generally accumulate some of osmolytes such as proline under salt and drought stress (Delauney & Verma, 1993), which protect the proteins against denaturation and also act as osmotic balancing agents (Sivakumar et al., 2000). Content of total free phenols was measured according to the method developed by Swain and Goldstein (1959) using Folin - Ciocalteau reagent. Caffeine acid was used as a standard. The absorbance was registered at 725 nm. Plants generally accumulate some of osmolytes such as proline under salt and drought stress, which protect the proteins against denaturation and also act as osmotic balancing agents (Chadalavada et al., 1994; Kavi Kishor et al., 2005). Proline content was determined by measuring the absorbance at 520 nm after reaction with a mixture glacial acetic acid and ninhydrin according the method of Bates et al., (1973). Proline concentration in the samples was determined from the standard curve calibrated with different concentrations of the standard proline.

Hydrogen peroxide is known to damage various cellular components and evoke structural modifications of proteins, lipids and DNA under stress (Halliwell & Gutteridge, 2002). Hydrogen peroxide and malondialdehyde contents are routinely estimated together with electrolyte leakage measurements to assess the extent of oxidative stress. The injurious impact of reactive oxygen species on the membranes of cells is realized by lipid peroxidation. The basic damage products by this process are aldehydes and mainly MDA. The accumulation of MDA is an especially sensitive marker of stress. For determination of the amount of MDA as a final product of lipid peroxidation, the method of Kramer et al. (1991) was used. The absorbance was measured at 532 nm and 600 nm. The content of endogenous free thiol groups was determined using the Elman's reactive according to the method of Edreva & Hadjiska, (1984). Hydrogen peroxide absorbance was measured at 390 nm after reaction with KJ according to Alexieva et al., (2001). Content of H_2O_2 was calculated using the standard curve with known amount of KJ. Glutathione content was measured according to Gronwald et al., (1998).

Compounds bearing a free thiol groups (-SH), such as low molecular cell metabolites like glutathione, as well as a number of enzymes, which are active only in a reduced state, play a key role in important cellular functions, and the massive oxidation of -SH groups could be regarded as an aspect of oxidative toxicity (Haugard, 2000). The relationship between the SH-state in plants and their resistance to various stress factors is well known. Glutathione is major fraction of the SH pool in the cells (Haugard, 2000; Noctor et al., 2002).

Pigment contents (chlorophyll a and b) were calculated after the extraction of leaf material with 80% acetone according to the method of Arnon (1949). The extinction of chlorophyll a and chlorophyll b was determined at 663 nm and 845 nm using a spectrometer Specol 11.

5. Spectral measurements

5.1 Leaf spectral reflectance

Hyperspectral reflectance data were collected in VIS and NIR spectral ranges (450-850 nm) by using a portable fibre-optics spectrometer USB2000 (http://www.OceanOptics.com). In the range investigated the main part of the reflected from leaves radiation is concentrated. Data were obtained at 1170 spectral bands with a step of 0.3 nm and a spectral resolution of 1.5 nm.

The reflectance measurements were carried out on an experimental setup in laboratory (Fig. 10). The entrance lens, a standard screen WS1 (diffuse reflectance white plastic with Lambertian reference surface) and plant leaves were set on a special adjustable platform, which provides an accurate relative positioning of all equipment components. The fibre-optic cable was located at nadir view (perpendicular to leaf surface). As a light source a halogen lamp providing homogeneous illumination of measured leaf surfaces was used. The acquisition and processing of data were carried out by means of portable computer using specialized software. The spectral reflectance characteristics were obtained as a ratio of the reflected radiation from the leaves and the reflected radiation from WS1 screen. The measurements were performed on fresh, immediately picked off soybean leaves at the stage of 4th trifoliate expanded leaf from up to 25 plants from each investigated group.
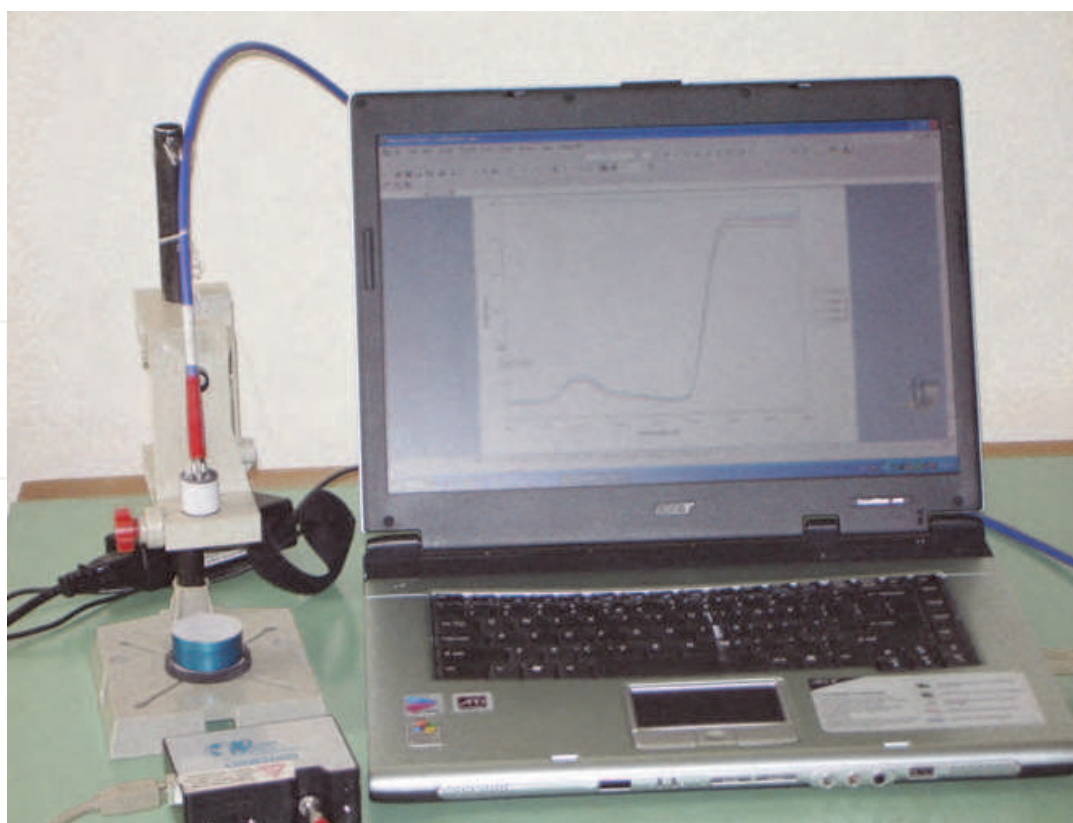

Fig. 10. Experimental setup for spectral reflectance measurements.

5.2 Chlorophyll fluorescence

The spectral measurements of the chlorophyll fluorescence were carried out under laboratory conditions using the same portable fibre-optics spectrometer (USB2000). Data were collected in the VIS and NIR spectral ranges (600-900) nm in 910 spectral bands with a step of 0.3 nm where the main part of the emitted from the plants fluorescence radiation is concentrated. As a source of actinic light, a LED diode with light output maximum at 470 nm and light intensity 507 μmol m^{-2} s^{-1} was used. The tested leaves were dark adapted before the measurements for ten minutes. The abaxial side of the leaves was irradiated with actinic light and the exited fluorescence was measured from the adbaxial leaf surface. The control of the spectrometer and the acquisition and processing of data were carried out by means of specialized software. The measurements were conducted on fresh detached leaves from by 20 plants of the each group of plants in the 4th trifoliate expanded leave node on the 14th day after the salinity treatment.

5.3 Statistical analysis

The hyperspectral reflectance and fluorescence data of the control and treated plants were subjected to statistical analysis through the Student's t-criterion and linear stepwise Discriminant Analysis (DA). Because 1170 reflectance and 910 fluorescence values were available to be used as classification features, it was computationally efficient to select a subset of bands on the basis of discriminant capability.

The reflectance analysis was performed in four most informative for investigated plants spectral ranges: green (520-580 nm, maximal reflectivity of green vegetation), red (640-680 nm, maximal chlorophyll absorption), red edge (680-720 nm, maximal slope of the reflectance spectra) and the NIR (720-770 nm) (Krezhova et al., 2005, 2007). The statistical significance of the differences between SRC of control and treated plants was examined in eight spectral bands (wavelengths) chosen to be disposed uniformly over the above mentioned ranges (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).

The fluorescence spectra were analyzed in five characteristic spectral bands, chosen at wavelengths: λ_1 (at the middle of the forefront edge), λ_2 (first maximum), λ_3 (at the middle between first and second maximum), λ_4 (second maximum), and λ_5 (at the middle of the rear slope). They are illustrated in Fig. 11 for a typical fluorescence spectrum of green vegetation.

The Student's t-criterion and linear DA were applied for determination of the statistically significance of differences at $p<0.05$ between the means of sets of the values of the reflectance and chlorophyll fluorescence of control and treated plants in the above mentioned wavelengths. They were further regarded as discriminative features. The Student's t-criterion was utilized under the prerequisite for the existence of numerous, independent and approximately of one and the same order factors of small impacts on the variables under examination. DA was used to increase classification accuracy. One output of the method is the determination of the posterior probability that spectral data of a given leaf belongs to the class of control or treated plants. For this purpose discriminant analysis will be implemented in one dimensional spaces defined by the features examined. In some of the cases DA was performed in two or three-dimensional spaces for enhancement of the discriminative possibility.
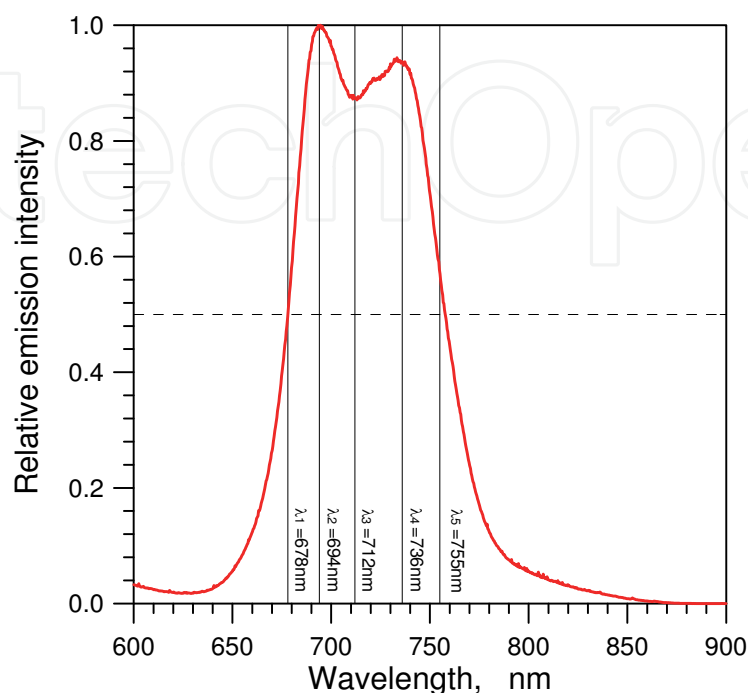
Fig. 11. Characteristic wavelengths chosen for statistical analysis of fluorescence.

All statistical analyses were conducted using the STATISTICA software, Version 6.1, 2002, (http://www.statsoft.com).

6. Results and discussion

6.1 Influence of a single stress factor at soybean plants

The effect of salinity on leaf spectral reflectance of soybean plants for the first part of the experiments (see Fig. 3) is shown in Fig. 12 (Krezhova et al., 2009a). The SRC were averaged over all studied areas (pixels) of leaves of control and treated with each of the two salt concentrations plants. The discrepancy (lack of coincidence or very small differences) between the characteristics of control and treated by 40 mM NaCl plants was observed in the green (520-580 nm, maximal chlorophyll reflection) and NIR ranges.

At 80 mM NaCl the values of the average SRC of treated leaves with respect to control decrease significantly in both the green and red (640-680 nm) ranges. In the red edge (680-720 nm) it is observed a shift to longer wavelengths (8 nm) indicating the occurrence of stress. Necrosis spots were seen on some of the leaves of plants treated with this NaCl concentration (Fig. 3 c). The red edge position changed significantly with the increase in NaCl concentration applied to the plants and it is a consequence of the decreased chlorophyll content determined by biochemical method. In the NIR range the reflectance changed non-significant against the control.
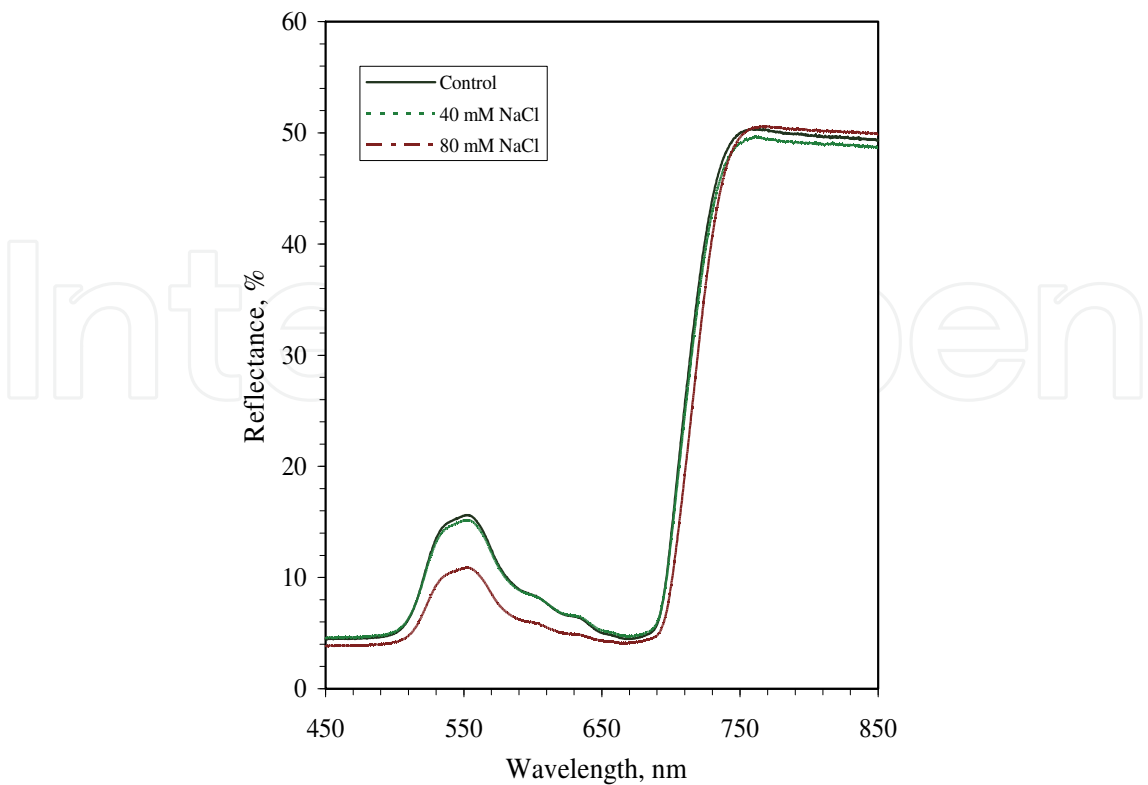
Fig. 12. Averaged spectral reflectance characteristics of control and treated with 40 mM NaCl and 80 mM NaCl soybean plants.

The results (p-level of the difference between SRC means of treated plants and SRC means control plants at a given λ) from the statistical analysis of the spectral data are set out in Table 1 and Table 2. In Table 1 p_{st} stands for the significance p-level of the Student's t-criterion. In Table 2 p_{DA} designates the significance p-level of the DA model. The index c stands for reflectance or fluorescence data of control plants. Statistically significant differences between SRC means of control and treated at 80 mM NaCl concentration were detected at $p<0.05$ by means of the Student's t-criterion in each wavelength with the exception of λ_8 in the NIR range. The impact of 40 mM NaCl salinity is not sufficient to provoke detectable changes in SRC in the wavelengths examined.

Student's t-criterion			
40 mM NaCl		80 mM NaCl	
Pairs compared	p_{st}	Pairs compared	p_{st}
λ_1/λ_{1c}	0.328	λ_1/λ_{1c}	<0.001
λ_2/λ_{2c}	0.210	λ_2/λ_{2c}	<0.001
λ_3/λ_{3c}	0.185	λ_3/λ_{3c}	<0.001
λ_4/λ_{4c}	0.061	λ_4/λ_{4c}	<0.001
λ_5/λ_{5c}	0.125	λ_5/λ_{5c}	<0.001
λ_6/λ_{6c}	0.120	λ_6/λ_{6c}	<0.001
λ_7/λ_{7c}	0.082	λ_7/λ_{7c}	<0.001
λ_8/λ_{8c}	0.285	λ_8/λ_{8c}	0.94

Table 1. Significance p-level of the Student's t-criterion in the cases of 40 mM NaCl and 80 mM NaCl salinity.

Similar results are obtained through linear DA. The grouping variable used on the first stage of DA implementation was designed to consist of only two groups - control and by one of the treated with different NaCl concentration plants. Since the DA significant p-level for the case of 40 mM NaCl turned out to be >>0.05 with all wavelengths (0.12<p<0.98) the p_{DA} are not shown in the Table 2. Anyway, if for example the three dimensional space (λ_4, λ_6, λ_7) is used, the p_{DA} level is p<0.001 while the incorrectly classified cases are 12 from 69.

Discriminant analysis, 80 mM NaCl		
One dimensional spaces	p_{DA}	Number of incorrectly classified cases
λ_1	<0.001	0
λ_2	<0.001	0
λ_3	<0.001	0
λ_4	<0.001	18 from 69
λ_5	<0.001	1 from 69
λ_6	<0.001	6 from 69
λ_7	<0.001	11 from 69
λ_8	0.95	29 from 69

Table 2. Significance p-level of the Discriminant analysis model in the case of 80 mM NaCl salinity.

The contents of the evaluated stress markers and chlorophyll a and b are shown in Table 3. The values of control plants were taken as 100%. The 40 mM NaCl treatment did not provoke changes in the phenol content in soybean leaves, while 80 mM NaCl caused a decrease of the content by about 33.6%. Such sharp decrease of phenols content in the leaves treated by 80 mM NaCl gives grounds to consider the phenols as playing the role of endogen antioxidant in plants.

Stress markers, pigments	Control	40 mM NaCl	80 mM NaCl
Phenols, (µM/gDW)	123.2 ±2.1	122.9 ±1.1 2%	81.8±0.9 33.6%
Proline, (nmol/gDW)	30.8±0.7	34.6±2.3 12.3%	48.1±1.9 56.2
MDA (nmol/g DW)	97.6±5.3	114.9±6.8 18%	140.2±5.6 44%
H_2O_2 (µM/gDW)	6.8±0.06	5.1±0.04 25%	6.3±0.02 7%
Thiol groups (µM/gDW)	2.49±0.01	3.59±0.04 42%	4.23±0.03 70%
Chlorophyll a (mg/g FW)	1.39±0.03	1.09±0.02 21%	0.71±0.03 49%
Chlorophyll b (mg/g FW)	0.60±0.01	0.46±0.03 23%	0.35±0.01 42%
GSSG/ TG (%)	52.4	64.1 23%	76.3 46%

Table 3. Values of the biochemical parameters of salinity treated soybean plants.

A number of authors have observed that most plant species exhibit a remarkable increase in their proline content in consequence of the action of different kinds of stress such as UV-radiation, drought, salinity, etc. (Sivakumar et al., 2000; Jogeswar et al., 2006; Sun et al., 2008). Characteristic changes in proline content at the salinity stress are described in roots and leaves of alfalfa and pea (Tramontano & Jouve, 1997), and in leaves of cotton and bean (Brankova et al., 2005). Our results show that an increase of proline content by about 12% takes place under the influence of salinity at the lower concentration. A more significant increase of proline by about 56% was observed at 80 mM NaCl concentration.

An increase in hydrogen peroxide and MDA contents upon salt stress has been reported for different plant spices (Yang et al., 2008). This increase was shown to be related to the amount of stress and well correlated with lipid membrane damage. Our results show that salinity at 40 mM NaCl leads to an increase of MDA by about 18% in comparison with the control. A more substantial increase is observed at 80 mM NaCl that reaches 44%. These results agree with the findings of Jogeswar et al. (2006) who have established a significant increase of MDA at treatment of sorghum (Sorghum bicolour) with 150 mM NaCl.

Unexpectedly, in our experiments the H_2O_2 content was found lower by about 25% at the low salinity and by about 7% at the high salinity. This finding provides grounds to continue our investigations in order to determine the activity of enzymes from the antioxidant system (catalase and peroxidase) using H_2O_2 as substrate.

The measurements of thiol groups observed an increase by about 40% at the treatment with 40 mM NaCl. The salinity at 80 mM NaCl lead to about doubling (by 70%) of the free -SH groups. By our opinion, the increase of thiol groups might serve as a marker of damages induced by salinity stress. The ratio of oxidized form glutathione to total glutathione (GSSG/TG) is much higher than the control at 80 mM NaCl which is evidence of a strong reduction of the capacity of antioxidant system for the plants under study.

The averaged fluorescence spectra over 20 leaves of the control and by 20 treated leaves with each of the two NaCl concentrations of the same soybean plants used in the first part of the experiments are shown in Fig. 13 (Iliev et al., 2009a). All spectra are normalized to their second maximum at λ_m which in this case coincided with the wavelength of 738 nm. Changes in the fluorescence spectra of treated plants against the control were predominantly observed in the arising forefront. Curve 2 (the averaged leaf spectrum of plants treated with 40 mM NaCl) slightly differs against control curve 1 in the spectral range 640-680 nm. Curve 3 (80 mM NaCl treatment) differs against curve 1 significantly within the spectral range 600-740 nm.

The Student's t-criterion and linear DA were applied to estimate the statistical significance of the differences between the means of the indices chosen to characterize the fluorescence spectrum (halfwidth, wavelength at the first maximum, area) defined as, (see Fig. 11):

- halfwidth of the fluorescence spectrum ($\lambda_5 - \lambda_1$); λ_1 (relative emission intensity, REI = 0.5 in the forefront) and λ_5 (REI = 0.5 in the rear slope);
- REI at wavelength $\lambda_2 = \lambda_{1m}$ (at first maximum of the fluorescence spectrum);
- fluorescence spectrum area S (between wavelengths 640 nm and 840 nm).

The main results of the statistical analysis are summarized in Table 4 and Table 5. It is seen in Table 4 that the changes of the indices under the conditions of 80 mM NaCl concentration could be detected at p<0.05 by means of each of the indices. Also, it is clear that the impact of 40 mM NaCl is not sufficient to induce detectable changes in any of the fluorescence indices.
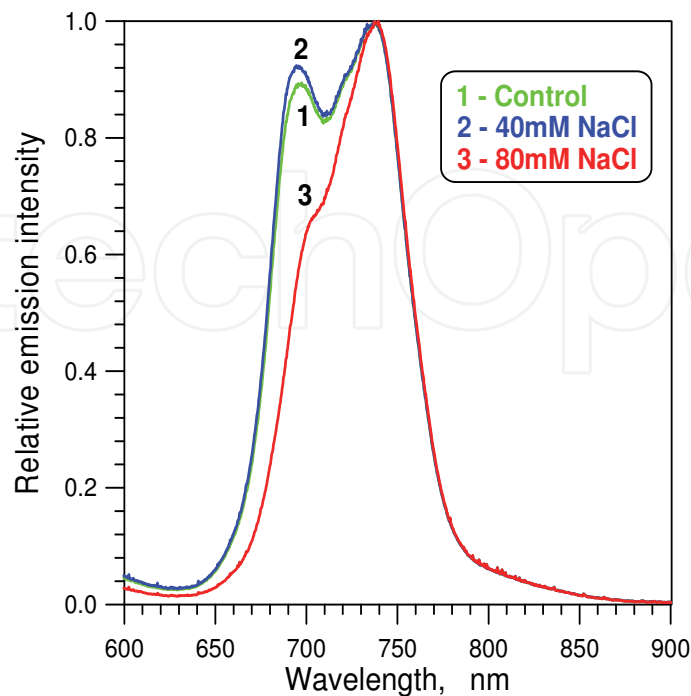
Fig. 13. Averaged fluorescence spectra of control and treated with: 40 mM NaCl and 80 mM NaCl soybean leaves.

Student's t-criterion			
Salinity 40 mM NaCl		Salinity 80 mM NaCl	
Pairs compared	p_{st}	Pairs compared	p_{st}
λ_1/λ_{1c}	0.955	λ_1/λ_{1c}	<0.001
$\lambda_{1m}/\lambda_{1mc}$	0.045	$\lambda_{1m}/\lambda_{1mc}$	0.007
λ_5/λ_{5c}	0.257	λ_5/λ_{5c}	0.009
$(\lambda_5-\lambda_1)/(\lambda_5-\lambda_1)_c$	0.202	$(\lambda_5-\lambda_1)/(\lambda_5-\lambda_1)_c$	<0.001
S/S_c	0.012	S/S_c	<0.001

Table 4. Significance p-level of the t-criterion for the set of fluorescence indices.

Similar results are obtained through linear discriminant analysis by making use of one dimensional spaces defined by each of the indices herein used, the p_{DA} level is p<0.05 while the incorrectly classified cases are not more than 5 cases from 24. The two indices λ_m and S also indicated perspective possibilities for detection of salinity injures on soybean plants.

The results from the chlorophyll fluorescence analysis revealed that the low NaCl concentration applied does not produce statistically significant changes in the leaf fluorescence. Applying of high NaCl concentration lead to significantly changed forefront of the fluorescence spectra due to caused salinity stress in the soybean plants.

In summary, the results from the first part of experiments have shown that there is a difference in the spectral reflectance characteristics in response to different salt

concentration treatment of soybean plants. The shift of the red edge position correlated with increased concentration of the salinity. Low NaCl concentration (40 mM) caused insignificant changes in the SRC and led to salinity tolerance whereas high NaCl concentration (80 mM) induced considerable SRC changes implying presence of salinity stress in soybean plants. This finding was in agreement with the outcome from the chlorophyll fluorescence analysis carried out on the same plants and evaluated biochemical stress markers such as phenols, proline, malondialdehyde, thiol groups, hydrogen peroxide, and leaf pigment contents (Chl a and Chl b).

Discriminant Analysis				
	Salinity 40 mM NaCl		Salinity 80 mM NaCl	
One dimensional spaces	p_{DA}	Number of incorrectly classified cases	p_{DA}	Number of incorrectly classified cases
λ_1	0.996		<0.001	0
λ_{1m}	0.044	5/22	0.0132	5 from 24
λ_5	0.256		0.019	3 from 24
$(\lambda_5 - \lambda_1)$	0.199		<0.001	1 from 24
S	0.0123	8/22	<0.001	0

Table 5. Significance p-level of the Discriminant analysis model for the set of fluorescence indices.
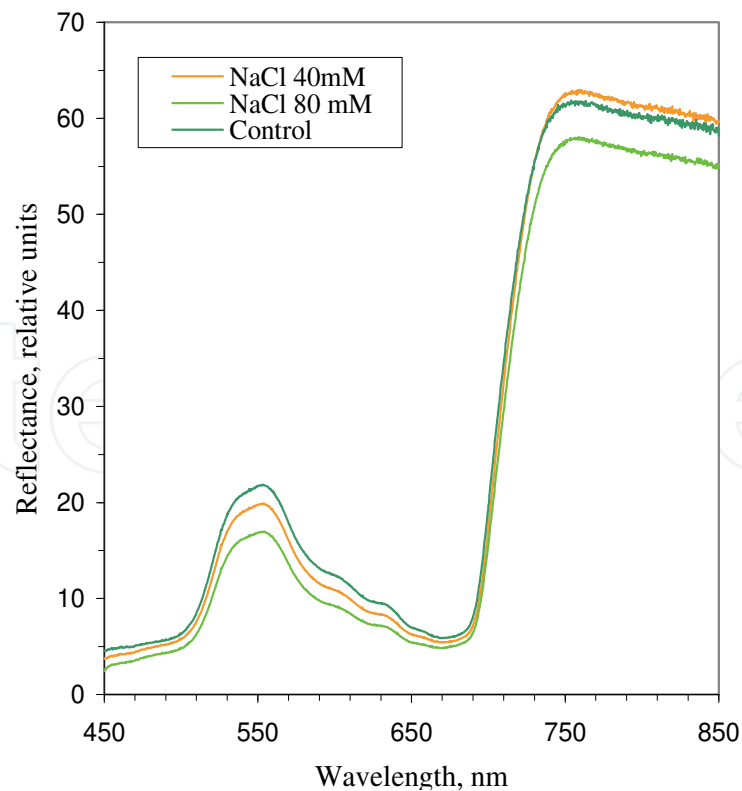

Fig. 14. Averaged spectral reflectance characteristics of control and treated with 40 mM and 80 mM NaCl nitrogen fixing soybean plants.

The second part of experiments was aimed at studying of the salinity effect on nitrogen fixing soybean plants. The measurements were performed on 25 areas (pixels) of randomly picked off leaves from each group of plants. The averaged SRC are displayed in Fig. 14 (Krezhova et al., 2009b). It is seen that the values of the SRC of the treated leaves with respect to control decrease significantly in both the green and red (450-680 nm) spectral ranges. In the red edge region it is observed a shift to longer wavelengths in correspondence with the increase in NaCl concentration applied, which is indicating occurrence of plant stress. For the case of 40 mM NaCl the shift is 2 nm while for the high salt concentration it is 6 nm. In the NIR range, the reflectance at low NaCl concentration increased while at high NaCl concentration it decreased due to the changes of water and nitrogen content in the leaves.

The results of application of the Student's t-criterion are presented in Table 6. Statistically significant are differences for which p<0.05 and only the differences in wavelengths λ_7 and λ_8 at 40 mM NaCl salinity are non-significant.

Pairs compared	40 mM NaCl		80 mM NaCl		Control
	p_{st}	mean	p_{st}	mean	mean
λ_1/λ_{1c}	<0.001	14.41	<0.001	12.10	15.47
λ_2/λ_{2c}	<0.001	18.96	<0.001	16.13	20.74
λ_3/λ_{3c}	<0.001	19.86	<0.001	16.93	21.74
λ_4/λ_{4c}	0.0027	5.46	<0.001	4.87	5.92
λ_5/λ_{5c}	<0.001	22.95	<0.001	20.76	25.22
λ_6/λ_{6c}	0.0298	44.98	<0.001	41.15	46.32
λ_7/λ_{7c}	0.3117	50.50	<0.001	46.27	51.15
λ_8/λ_{8c}	0.3048	62.61	0.001	57.98	61.76

Table 6. Significance p-level of the t-criterion in the case of 40 mM NaCl and 80 mM NaCl salinity.

Linear DA was implemented making use of one-dimensional spaces defined by each one of the wavelengths. Table 7 shows that probability levels p_{DA} coincide with that of the Student's t-criterion as the grouping variable consisted for each concentration of only two classes: control and treated plants (40 mM NaCl or 80 mM NaCl) at a given wavelength. At λ_7 and λ_8 (the same as for the t-criterion) statistically significant differences between SRC means for control and tread plants were not observed. Therefore the number of incorrectly classified cases was maximal at these wavelengths.

To illustrate better discriminative DA possibilities we performed DA in a two-dimensional space defined by the wavelengths λ_7 and λ_8, which manifested worst results when applied separately. Making use of data for concentration 40mM NaCl the p-level turned out to be <0.001 and the number of incorrectly classified cases was only 12.

The contents of the evaluated biochemical parameters - stress markers (phenols, proline, MDA, H_2O_2, free thiol groups, ratio of oxidized to total glutathione GSSG/TG) and content of chlorophyll a and b, are shown in Table 8. The values for control plants were taken as 100%. The 40 mM NaCl concentration lead to a phenol content decrease of about 19%, whereas the 80 mM NaCl salinity treatment provoked their much stronger decrease (by 59%). At 40 mM NaCl the proline content increased with 8%. A considerable increase of the proline (73%) was observed at 80 mM NaCl concentration. Under salinity stress most plant

One dimensional spaces	40 mM NaCl p_{DA}	40 mM NaCl Number of incorrectly classified objects	80 mM NaCl p_{DA}	80 mM NaCl Number of incorrectly classified objects
λ_1	<0.001	14 from 50	<0.001	5 from 50
λ_2	<0.001	13 from 50	<0.001	5 from 50
λ_3	<0.001	13 from 50	<0.001	6 from 50
λ_4	0.0027	15 from 50	<0.001	7 from 50
λ_5	<0.001	12 from 50	<0.001	7 from 50
λ_6	0.0298	21 from 50	<0.001	8 from 50
λ_7	0.3117	22 from 50	<0.001	10 from 50
λ_8	0.3048	24 from 50	0.001	15 from 50

Table 7. Significance p-level of the linear DA in the case of 40 mM NaCl and 80 mM NaCl salinity of nitrogen fixing soybean plants.

species exhibit a remarkable increase in the proline content. MDA is an indicator of free radical production and potential to withstand and recover after membrane injury under stress. In our experiment, we established that 40 mM NaCl salinity induced a reduction of the MDA content with 17%, while 80 mM NaCl salinity lead to a decrease of the MDA content with 55%. When measuring the thiol groups, an increase of about 108% of their content was observed at concentration 40 mM NaCl. A much higher free thiol groups' content increase of 151% was established at concentration 80 mM NaCl. The enlargement of the content of free thiol groups is a marker for the presence of injures caused by salinity stress. The H_2O_2 levels in our experiment indicate that under the conditions of salinity stress the H_2O_2 content becomes larger; it became 126% at the high salt concentration of 80 mM NaCl. After salinity treatment of soybean plants the ratio of oxidized to total glutathione GSSG/TG increased with approximately 13% at the lower NaCl concentration and of the order of 26 % at the higher concentration. This brings to decreasing of the nitrogen fixing capacity and the plant sustainability.

Stress markers, pigments	Control	40 mM NaCl		80 mM NaCl	
Phenols, μmol/gDW	131.3 ±2.1	106.7 ±1.1	81% (19↓)	54.6±0.9	41.6% (59↓)
Proline, nmol/gDW	21.9±0.7	23.7±2.3	108% (8↑)	37.9±1.9	173% (73↑)
MDA, nmol/gDW	141.3±5.3	117.5±6.8	83.1% (17%↓)	62.7±5.6	44.3% (55↓)
H_2O_2 , μmol/gDW	2.8±0.06	3.41±0.04	121% (21↑)	6.35±0.02	226% (126↑)
Thiol Groups, μmol/gDW	1.03±0.02	2.15±0.07	(108% ↑)	2.59±0.08	(151%↑)
Chlorophyll a, mg/g FW	1.22±0.03	1.19±0.02	98%(2↓)	0.94±0.03	79% (21↓)
Chlorophyll b, mg/g FW	0.54±0.01	0.38±0.03	70.3%(30↓)	0.34±0.01	63% (37↓)
GSSG/TG, %	57.2	64.6	113%(13↑)	21.3	126% (26↑)

Table 8. Values of the biochemical parameters of nitrogen fixing soybean plants.

The decrease of the leaf chlorophyll content under salinity stress is a main phenomenon of the plant sensitivity. In our experiments, both the content of chlorophyll a and chlorophyll b decreased at the two salinity levels, the decrease under 80 mM NaCl being larger.
Concluding, it was found that the results from the implementation of the two methods, leaf spectral reflectance and biochemical analysis, revealed that both the NaCl concentrations

bring to salinity stress in the nitrogen fixing soybean plants and to decline of the biological nitrogen fixation. The red edge shift to longer wavelengths is an indicator of stress and is correlated with the decreased chlorophyll content and salinity rate.

The course of the averaged fluorescence spectra over 20 control and 20 salinity treated leaves of nitrogen fixing soybean plants used for the second part of the experiments is shown in Fig. 15 (Iliev et al., 2009b). All spectra were normalized against their second maximum. Changes in the spectra of treated leaves against the controls were significant in the forefront and in the spectral range between first and second maximums (680-740 nm).
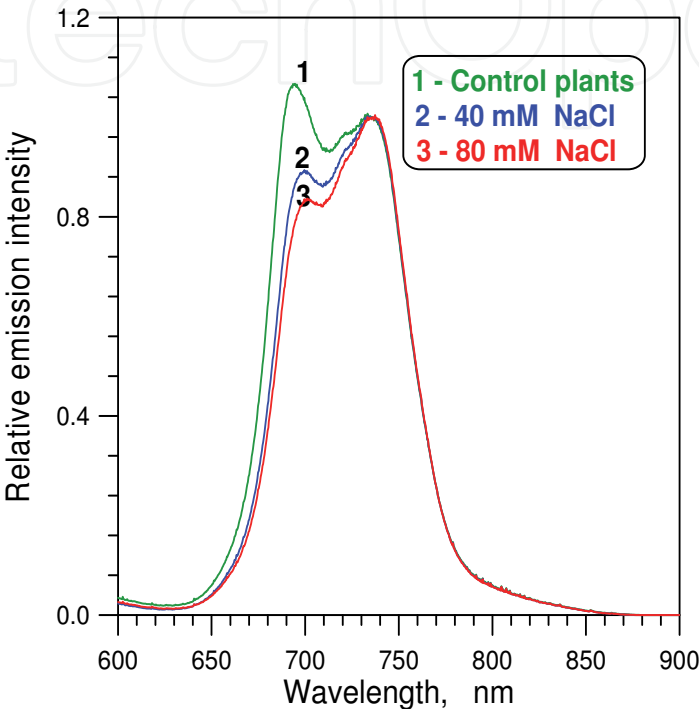

Fig. 15. Averaged fluorescence spectra of control and treated with 40 mM and 80 mM NaCl nitrogen fixing soybean plants.

The results of the Student's t-criterion and linear DA are displayed in Table 9 and Table 10. For analysis, five fluorescence values for each spectrum (from all 910) in characteristic wavelengths in the spectral range 600-900 nm were selected: λ_1 (at the middle of the forefront), λ_2 (first maximum), λ_3 (at the middle between first and second maximum), λ_4 (second maximum) and λ_5 (at the middle of rear slope), see Fig. 11.

Pairs	40 mM NaCl	40 mM NaCl	80 mM NaCl	80 mM NaCl	Control
compared	p_{st}	mean	p_{st}	mean	mean
λ_1/λ_{1c}	<0.001	911.6	<0.001	758.1	1345.8
λ_2/λ_{2c}	<0.001	2100.6	<0.001	1878.4	2672.0
λ_3/λ_{3c}	0.0016	2097.0	<0.001	1994.9	2332.9
λ_4/λ_{4c}	0.318	2436.9	0.2305	2408.1	2506.8
λ_5/λ_{5c}	0.480	1494.2	0.4919	1490.1	1525.1

Table 9. Significance p-level of the t-criterion for the set of amplitudes of the fluorescence spectra.

Statistically significant differences between data means at wavelengths λ_i and λ_{ic}, i = 1,…, 5 were established by the Student's t-criterion at p<0.05 for the data at the first three wavelengths and for both the NaCl concentrations. DA confirmed these findings in one dimensional spaces defined separately by each of the five wavelengths.

One dimensional spaces	40 mM NaCl p_{DA}	40 mM NaCl Number of incorrectly classified objects	80 mM NaCl p_{DA}	80 mM NaCl Number of incorrectly classified objects
λ_1	<0.001	8 from 40	<0.001	4 from 34
λ_2	<0.001	10 from 40	<0.001	5 from 34
λ_3	0.0016	11 from 40	<0.001	8 from 34
λ_4	0.318	15 from 40	0.2305	14 from 34
λ_5	0.480	18 from 40	0.4919	12 from 34

Table 10. Significance p-level of the linear DA for the set of amplitudes of the fluorescence spectra.

The results revealed that the two NaCl concentrations applied produce statistically significant changes in the forefront of leaf fluorescence spectra of the nitrogen fixing soybean plants. This corresponds to the salinity stress disclosed by the biochemical parameters (stress markers and pigments) and by the spectral reflectance in the VIS and NIR spectral ranges evaluated for the same soybean plants. The two remote sensing techniques (chlorophyll fluorescence and spectral reflectance) independently detected that both the NaCl concentrations bring to salinity stress in the nitrogen fixing soybean plants.

6.2 Influence of the combined impact of stresses on soybean plants

The third part of experiments aimed at assessing of the impact of the single stress factor salinity and combined stress factors salinity and enhanced UV-B radiation on young nitrogen fixing soybean plants. Spectral data were taken for 25 areas (pixels) of leaves from each group of investigated plants. Fig. 16 shows the averaged spectral reflectance characteristics of the control group and salinity treated at two NaCl concentrations nitrogen fixing soybean plants (Krezhova et al., 2011a). It is seen that changes in SRC for the salinized plants are noticeable in all investigated spectral ranges. The effect of the salinity was manifested by decreasing of the values in the green (520-580 nm) and red (640-680 nm) ranges and increasing in the NIR (720-770 nm). The red edge position of the SRC at low and high NaCl treatments is shifted to longer wavelengths (1 nm and 5 nm, respectively). On some of the leaves treated with 80 mM NaCl concentration the necroses spots were observed (Fig. 9a).

The results from the statistical analysis applying Student's t-criterion are shown in Table 11. Statistically significant differences are obtained at p<0.05 in the two cases of salinity treatment with the exception of 40 mM NaCl concentration in λ_6 and λ_8, and 80 mM NaCl in λ_7.
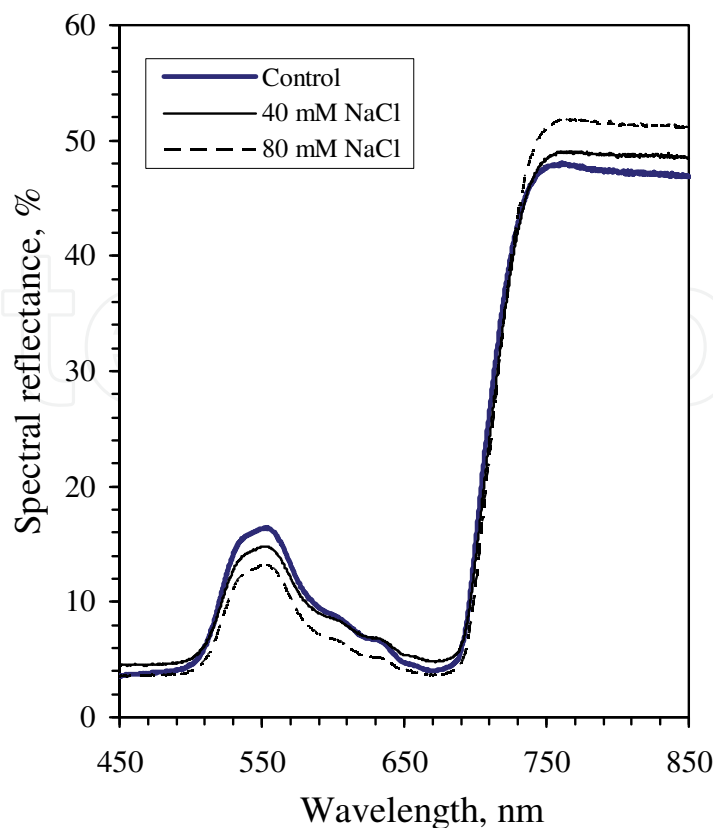
Fig. 16. Averaged spectral reflectance characteristics of control and treated with 40 mM and 80 mM NaCl nitrogen fixing soybean plants.

Pairs compared	Control mean	40 mM NaCl p_{St}	40 mM NaCl mean	80 mM NaCl p_{St}	80 mM NaCl mean
λ_1/λ_{1c}	11.93	<0,001	10.92	<0.001	9.28
λ_2/λ_{2c}	15.74	<0.001	14.21	<0.001	12.61
λ_3/λ_{3c}	16.40	<0.001	14.83	<0.001	13.18
λ_4/λ_{4c}	4.08	<0.001	4.83	<0.001	3.65
λ_5/λ_{5c}	19.27	<0.001	17.23	<0.001	15.37
λ_6/λ_{6c}	35.59	0.029	33.59	0.001	33.60
λ_7/λ_{7c}	39.47	0.011	38.06	0.167	38.79
λ_8/λ_{8c}	47.91	0.152	48.88	0.007	51.73

Table 11. Significance p-level of the t-criterion in the cases of 40 mm NaCl and 80 mm NaCl salinity.

Fig. 17 shows the averaged SRC of leaves of plants from the second set of three groups including the control (treated only with UV-B radiation) and the other two groups on which the combined action of stresses, salinity at two concentrations + UV-B radiation, was applied. The values of the averaged spectral characteristics of treated leaves with respect to control decrease significantly in the green and red (520-660 nm), and NIR ranges. For these SRC it is observed an approaching of the red edge position nearer to the control (2 nm), which is an indicator for diminishing effect of the salinity stress. Averaged SRC after (80 mM NaCl + UV-B) treatment is very close to the one after (40 mM NaCl + UV-B) treatment.

The results from the statistical analysis concerning the combined impact of stresses to soybean plants are set out in Table 12. They indicate the increasing number of wavelengths in which the differences of the SRC against the control are non-significant. In the case of (40 mM NaCl + UV-B) the differences are non-significant in the whole NIR range. For (80 mM NaCl + UV-B) treatment they are non-significant in four of the wavelengths.
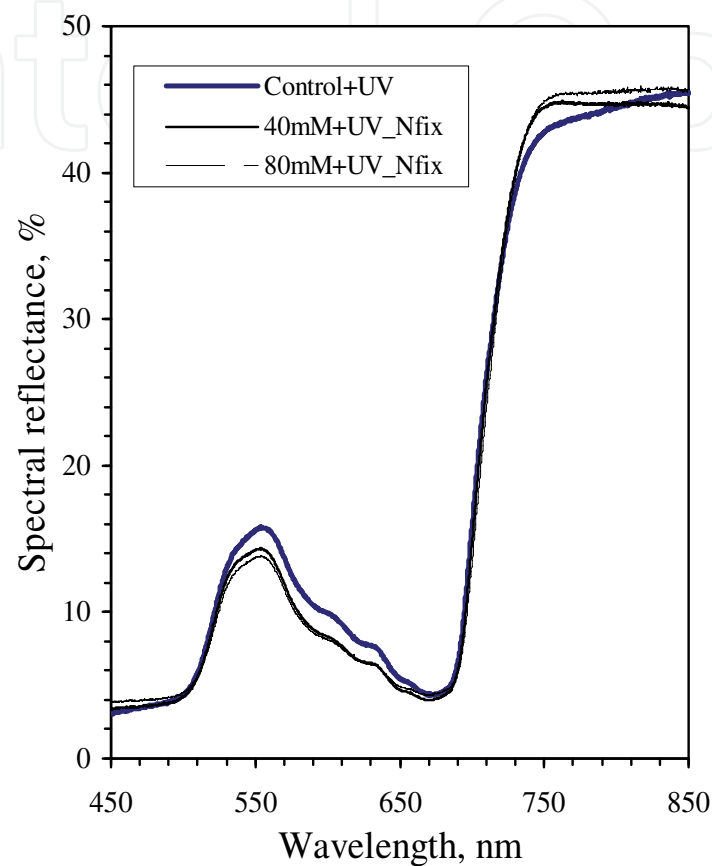

Fig. 17. Averaged spectral reflectance characteristics of control and treated nitrogen fixing soybean plants with: 40 mM NaCl + UV-B and 80 mM NaCl + UV-B.

Pairs compared	Control mean	40 mM NaCl +UV-B		80 mM NaCl + UV-B	
		p_{St}	mean	p_{St}	mean
λ_1/λ_{1c}	11.03	<0.001	10.38	<0.001	9.92
λ_2/λ_{2c}	14.63	<0.001	13.60	<0.001	13.06
λ_3/λ_{3c}	15.47	<0.001	14.30	<0.001	13.80
λ_4/λ_{4c}	4.41	<0.001	3.99	0.541	4.34
λ_5/λ_{5c}	19.79	0.006	19.71	<0.001	17.08
λ_6/λ_{6c}	33.09	0.733	33.37	0.067	32.23
λ_7/λ_{7c}	35.99	0.344	36.87	0.835	36.09
λ_8/λ_{8c}	43.22	0.214	44.80	0.102	45.29

Table 12. Significance p-level of the t-criterion in the cases of (40 mM NaCl +UV-B) and (80 mM NaCl + UV-B) treatment.

The effect of combined stress action of salinity + UV-B radiation was assessed by biochemical parameters: phenols, proline, malondialdehyde, and hydrogen peroxide. The contents of the evaluated stress markers are shown in Table 13.

The content of phenols decreased with 34.2% and 17% for low and high NaCl concentration, respectively. This is an indicator for decreasing of the action of salinity stress on the plants from the group treated with 80 mM NaCl +UV-B. The production of the MDA is a very sensitive stress marker. When its content is decreased it is a sign of the induction of sustainability to the applied stress. The increase in H_2O_2 and MDA contents was shown to be related to the amount of stress and well correlated with lipid membrane damage (Demiral & Turkan, 2005). In our experiment the amount of MDA increased for 40 mM NaCl + UV-B treatment with 4.5% and decreased in the other case with 7.6%. The hydrogen peroxide levels indicate that under the action of salinity stress + UV-B radiation the H_2O_2 content becomes higher with the increasing NaCl concentration. Hydrogen peroxide is known to damage cellular components and provoke structural modifications of proteins and lipids (Mandhania et al., 2006). Our experiment shows that a considerable increase of proline content takes place under the influence of salinity + UV-B at the lower concentration. By contrast, at 80 mM NaCl a significant decrease of proline by about 10.4% was observed.

Stress Markers, Pigments	Control +UV-B	40 mM NaCl + UV-B	80 mM NaCl +UV-B
Phenols (μM/g DW)	5.173 ±0.07	3.442±0.9 34.2%	4.289 ±0.04 17%
Proline (nmol/g DW)	8.942±0.87	22.955±1.9 156.7%	8.017±0.66 10.4%
MDA (nmol/g DW)	24.68±0.3	25.78±0.82 4.5%	22.80±0.66 7.6%
H_2O_2 (μM/g DW)	8.752±0.06	20.528±0.04 134.5%	50.903±0.02 481.6%

Table 13. Values of the biochemical parameters for the combined treatment of nitrogen fixing soybean plants.

Spectral data analysis revealed that in the case of the action of a single salinity stress on the young nitrogen fixing soybean plants there were statistically significant differences between the reflectance spectra of the leaves of control and treated plants at the two NaCl concentrations in all of the ranges examined with the exception of two wavelengths in NIR at 40 mM NaCl concentration and one wavelength at 80 mM NaCl concentration. In the SRC this effect was manifested by decreasing of the values in the green and red ranges and increasing in NIR. The results indicated that the plants were under conditions of stress which has been better pronounced for the higher NaCl concentration where necrotic and chlorotic lesions on the leaves have appeared due to chlorophyll degradation. This finding was established by evaluated biochemical markers of stress: phenols, proline, MDA, and H_2O_2.

In the case of treatment with (40 mm NaCl + UV-B) and (80 mm NaCl + UV-B) the results indicated that stress in the plants was also present but the influence of UV-B radiation after salinity reduces the consequences of salinity stress especially at the higher NaCl concentration. In the spectral reflectance characteristics this effect is manifested through an

increase of SRC values for treated plants and their approaching to the control SRC. This finding was established by the analysis of the evaluated biochemical stress markers.

The averaged fluorescence spectra of leaves of the same nitrogen fixing soybean plants treated with the two NaCl concentrations are displayed in Fig. 18 (Krezhova et al., 2011b). All spectra are normalized to their second maximum, which in this case is at $\lambda_4 = \lambda_m = 738$ nm. Changes in the spectra of treated plants (second and third groups) against the control (first group) increased with increasing NaCl amount and were observed in the spectral range spanning from arising forefront to the second maximum (640-740 nm). At their rear slope, the fluorescence spectra were with almost equal values.
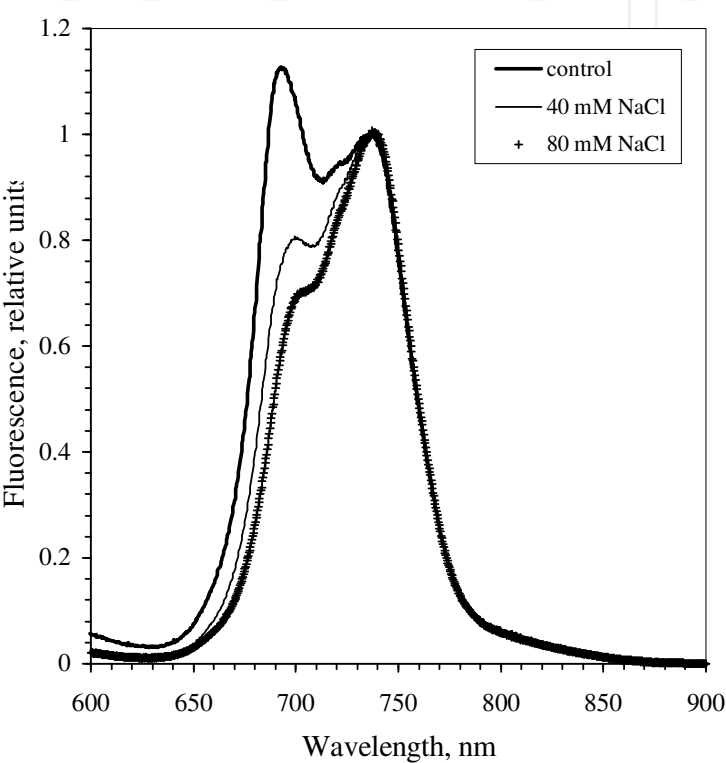

Fig. 18. Normalized average fluorescence spectra of control and treated with 40 mM NaCl and 80 mM NaCl nitrogen fixing soybean plants.

The results from the statistical analysis are presented in Table 14. Statistically significant differences were evaluated by Student's t-criterion at p<0.05 for the data at first three wavelengths for the two NaCl concentrations.

Pairs compared	Control mean	40 mM NaCl		80 mM NaCl	
		p_{St}	mean	p_{St}	mean
λ_1/λ_{1c}	0.335	<0.001	0.565	<0.001	0.221
λ_2/λ_{2c}	1.122	<0.001	0.770	<0.001	0.613
λ_3/λ_{3c}	0.912	<0.001	0.803	<0.001	0.735
λ_5/λ_{5c}	0.610	0.987	0.630	0.874	0.610

Table 14. Significance p-level of the t-criterion in the cases of 40 mM NaCl and 80 mM NaCl salinity of nitrogen fixing soybean plants.

The averaged normalized fluorescence spectra of the nitrogen fixing soybean plants subjected to combined stress factors (salinity + UV-B radiation) for the two NaCl concentrations are displayed in Fig. 19. It is observed that the differences between the spectra are less pronounced than those at single salinity stress impact. For the combined action of (40 mM NaCl+UV-B) the averaged spectrum has higher values than control due to induced tolerance to salinity stress from the action of UV-B radiation. For (80 mM NaCl+UV-B) treatment the values are lower and more close-set to the control due to the positive action of UV-B radiation expressed through the decreasing effect of salinity.
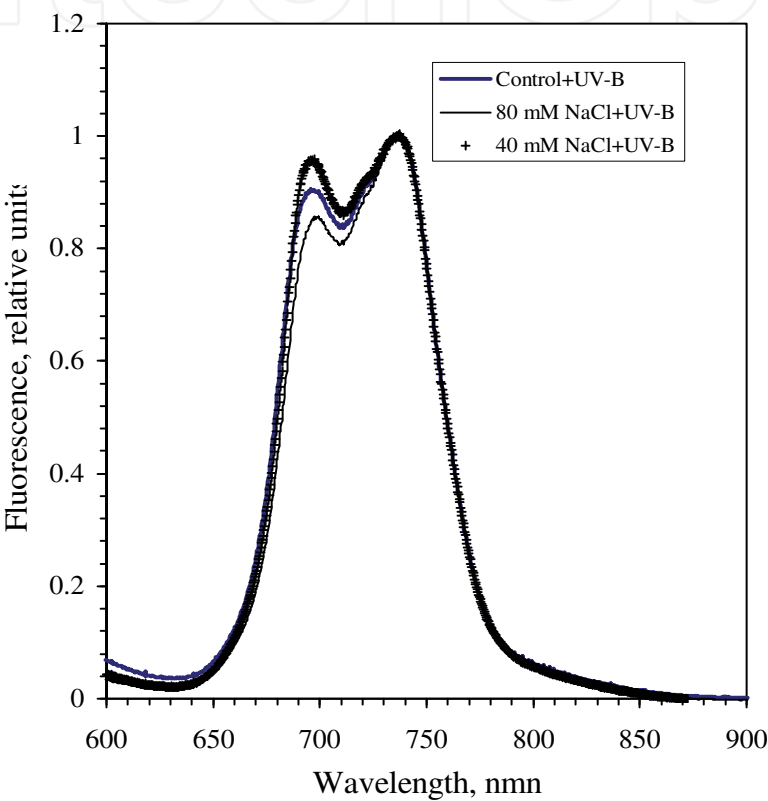

Fig. 19. Normalized average fluorescence spectra of control and treated with (40 mM NaCl + UV-B) and (80 mM NaCl + UV-B) nitrogen fixing soybean plants.

Table 15 shows the results from the Student's t-criterion for the combined stress treatment of the soybean plants. The differences against the control spectra are statistically significant at two of the investigated wavelengths for the two cases.

The results revealed that the salinity treatment at two NaCl concentrations significantly changed the fluorescence spectra of the nitrogen fixing soybean plants in the spectral range spanning from arising forefront to the second maximum (640-740 nm) due to salinity stress caused in the plants. In the case of initial salinity treatment of the plants followed by their irradiation with UV-B light the differences between the fluorescence spectra decreased due to the favourable effect of the UV-B light. These results were in compliance with the findings concerning the leaf spectral reflectance and biochemical parameters measured on the same nitrogen fixing soybean plants, treated with 40 mM and 80 mM NaCl concentrations and salinity + UV-B radiation.

Pairs compared	Control +UV-B mean	p_{St}	40 mM NaCl+UV-B mean	p_{St}	80 mM NaCl+UV-B mean
λ_1/λ_{1c}	0.440	0.967	0.441	0.832	0.380
λ_2/λ_{2c}	0.878	0.028	0.985	0.039	0.821
λ_3/λ_{3c}	0.836	0.010	0.873	0.041	0.811
λ_5/λ_{5c}	0.616	0.853	0.615	0.789	0.621

Table 15. Significance p-level of the t-criterion in the cases of 40 mM NaCl+UV-B and 80 mM NaCl+UV-B treatment for nitrogen fixing soybean plants.

7. Conclusions

In general this chapter illustrates the capability of hyperspectral remote sensing technologies in environmental stress studies and for timely making informed decisions in vegetation management. Our results demonstrate the potential of hyperspectral remote sensing methods, spectral reflectance and chlorophyll fluorescence in particular, for detection, discrimination and assessment of the effects of single and combined environmental stresses (salinity and enhanced UV-B radiation). A comparative analysis was performed between the changes of the leaf spectral reflectance characteristics, fluorescence spectra and values of the stress markers (phenols, malondialdehyde, thiol groups, proline, hydrogen peroxide), and chlorophyll content that were estimated by biochemical methods. As a result we obtained accurate and complementary benchmarking for plant responses to the environmental stress investigated.

The research and technological advances in the field of remote sensing have greatly improved the ability to detect and quantify environmental stresses that affect the productivity of agricultural vegetation. Hyperspectral remote sensing has made big progress with the advance of technique that has also increased the demand of its application for conducting, easily and without damage, rapid health condition assessments of vegetation cover. Further progress can be expected through extension of the inter-comparison of techniques, the parallel refinement of experimentally derived approaches and modelling, and by defining the optimum strategies reflecting different user requirements for scaling methods up to the canopy level. Modern management of agricultural resources is a complex endeavour that is now benefiting from a convergence of technical advances in information sciences, geographic positioning capabilities, and remote sensing systems. Using hyperspectral remote sensing as a tool for precision agriculture is a new field of research. Future work is necessary to further explore the full potential of this technology. The more programs and projects conducted in the recent years, the more models developed or improved for hyperspectral data processing to promote its applications.

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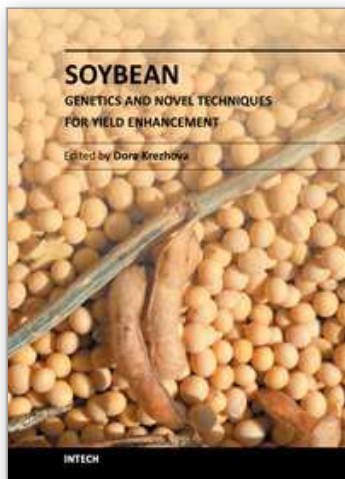
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This book presents the importance of applying of novel genetics and breading technologies. The efficient genotype selections and gene transformations provide for generation of new and improved soybean cultivars, resistant to disease and environmental stresses. The book introduces also a few recent modern techniques and technologies for detection of plant stress and characterization of biomaterials as well as for processing of soybean food and oil products.

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