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Abiotic Stress Diagnosis via Laser Induced Chlorophyll Fluorescence Analysis in Plants for Biofuel

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1. Introduction

In the past few decades there has been a widespread scientific and technological interest in laser-induced remote techniques to monitor the status of terrestrial vegetation (Svanberg, 1995). The most employed nowadays are those which exploit the fluorescence emission from the plant leaves generated in the photosynthesis process. The fluorescence of terrestrial vegetation consists almost exclusively of the fluorescence of leaves, which account for the largest surface of plants above ground. A small part of the absorbed light energy in the photosynthesis process is lost during the migration from the pigment antenna to the reaction centers and are dissipated by a number of non-photochemical processes, including heat, and re-emission of a small but easily detectable amount (2-5% *in vivo*) of the absorbed radiation. This re-emission occurs at longer wavelengths in the red and far-red spectral regions and is termed as Chlorophyll Fluorescence (ChlF) (Shreiber, 1983; Backer & Bradbury, 1981). Chlorophyll fluorescence represents an intrinsic signal emitted by plants that can be employed to monitor their physiological state including changes of the photosynthetic apparatus, developmental processes of leaves, state of health, stress events, stress tolerance, and also to detect diseases or nutrient deficiency of plants. In particular, the application of laser induced chlorophyll fluorescence spectroscopy has drawn much attention recently owing to the non-invasive and nondestructive nature of the technique (Svanberg, 1995; Lang & Lichtenthaler, 1991; Chappelle et al., 1984). The technique can be applied for chlorophyll level monitoring in basic photosynthesis research, agriculture, horticulture, and forestry. Abiotic stress (water deficit, salinity, heat, heavy metals soil contamination, intense light, etc) affects significantly crop growth and yield in agricultural areas all over the world. Thus, it is imperative to study their effect upon the crops and discriminate among abiotic stresses using new noninvasive and nondestructive remote sensing precision diagnostic techniques. These procedures allow one to employ intervention measures that will prevent damage to the crop and will not provoke economical losses. Our aim here, is to exploit laser-induced fluorescence signatures from plants to evaluate the effect of abiotic stresses (water

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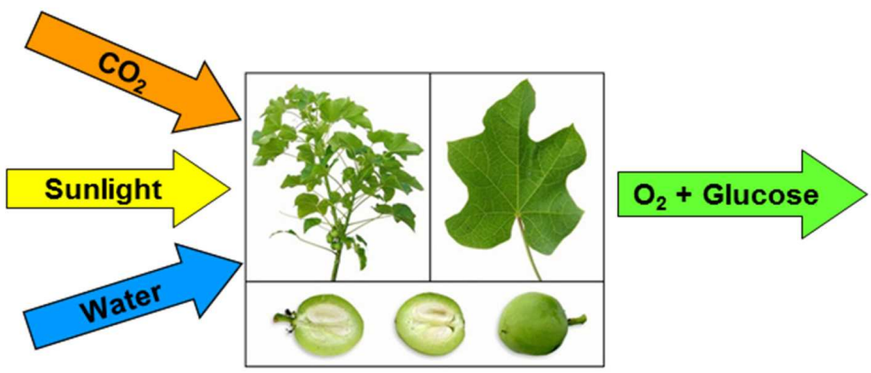
deficit and soil salinity) upon the evolution and characteristics of *in vivo* chlorophyll emission spectra of leaves of *Saccharum officinarum* and *Jatropha curcas* L. plants. The main interest in these plants species resides in the fact that they are used for large scale biofuel production in Brazil. Brazil is the world's second largest producer of ethanol fuel and the world's largest exporter. Together, Brazil and the United States lead the industrial production of ethanol fuel, accounting together for 89% of the world's production in 2009. Besides, *Jatropha curcas* L. plant is considered the poster child among many proponents to biodiesel production.

2. Photosynthesis

Photosynthesis is a biophotonic mechanism by which green plants exploit solar energy to reduce CO_2 and oxidize H_2O, as indicated in the process pictured in Fig. 1. Within the plant tissue, visible and near-infrared (NIR) light is absorbed (>80%) by photosynthetic pigments (Chlorophyll *a*, *b*, and carotenoids) and used to drive photosynthetic light reactions and associated electron transport reactions to reduce carbon and oxidize water in the Calvin cycle (Allen, 1992). Photosynthesis occurs in the chloroplasts where the photosynthetic pigments occupy. Chlorophyll molecules organized into two groups of pigments called photosystem I (PSI) and photosystem II (PSII), each containing "antennae" chlorophyll molecules and a central chlorophyll molecule (P680 and P700). The numbers are associated with the wavelengths corresponding to the maxima of the absorption spectra of the two species of Chlorophyll *a*.



$$6CO_2 + 12H_2O + \text{photons} + \text{chlorophyll} \rightarrow C_6H_{12}O_6 + 6H_2O + 6O_2 + \text{photons}$$

Fig. 1. Photosynthesis process in green plants

The physical mechanism of light energy absorption and migration to the reaction center is better visualized using the energy level and block diagram of Fig. 2.
The pigments antenna absorb much of the visible portion of the electro-magnetic spectrum, mainly in the near UV-blue region, as can be seen in the absorption spectra shown in Fig. 3.
There exist a very strong energy transfer mechanism taking place among pigment antenna where the light energy absorbed by carotenoids and chlorophyll *b* pigments resonantly transfer their energy to neighbours chlorophyll *a* molecules and the total energy is conveyed to reaction centers in which the migration process will occur as pictured in Fig. 2 (top). The chlorophyll fluorescence re-emitted light occurs in the red around 680-690 nm and far-red

730-740 nm spectral regions (Lang & Lichtenthaler, 1991; Chappelle et al., 1984). When excited with either UV or blue radiation, plants exhibit a fluorescence emission spectrum in

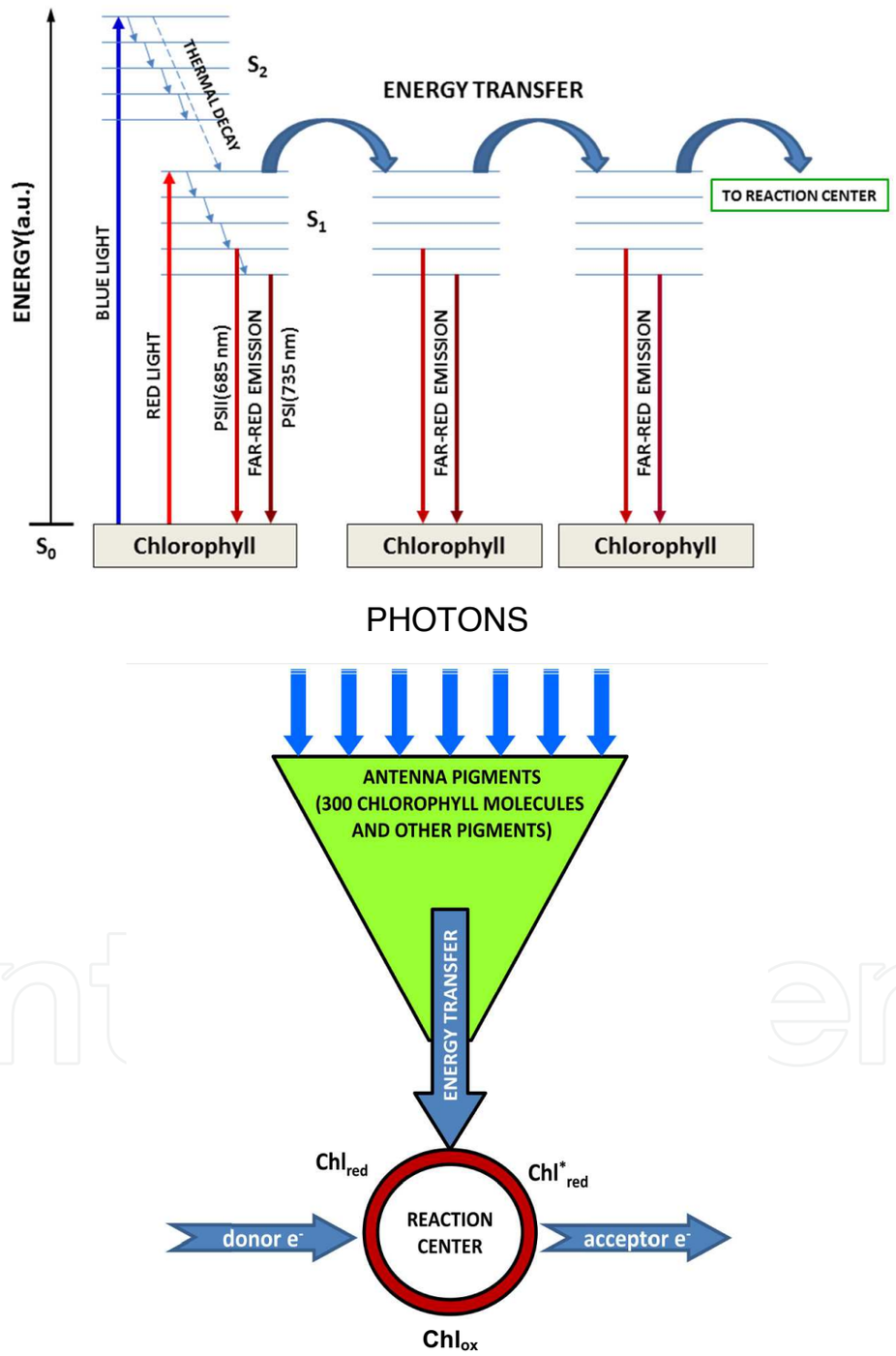


Fig. 2. Energy transfer (top) and light energy funneled to the reaction center (bottom)

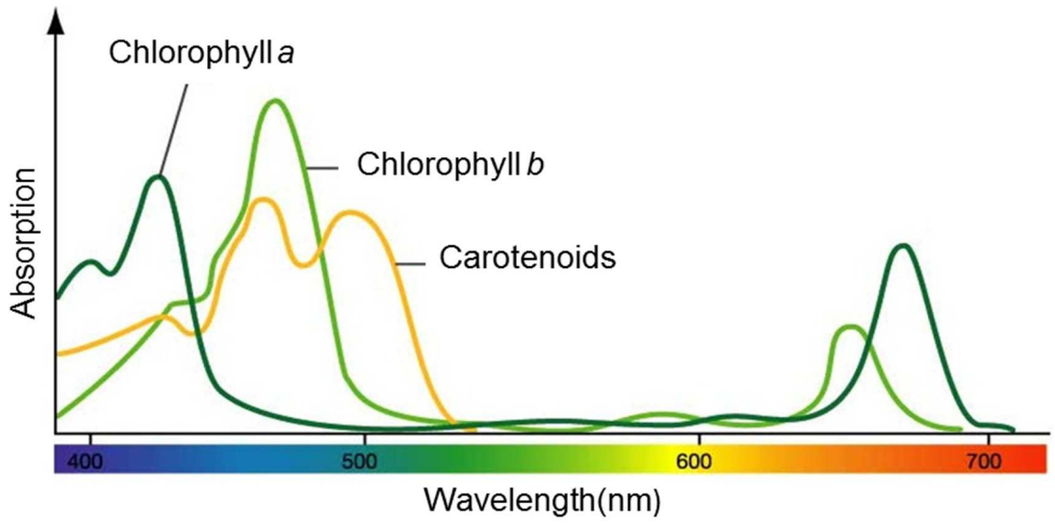


Fig. 3. Absorption spectrum of pigments antenna of green leaves

two distinct spectral regions blue-green (400-550 nm) and red-far-red (650-800 nm). However, in our case the fluorescence intensity of the blue-green spectral region was too small to be used as reliable chlorophyll fluorescence signatures, and was detected only in extreme cases, i.e., in plants under high degree of stress damage. On the other hand, the red fluorescence is characterized by a maximum in the red region (680-700 nm) which is attributted to the PSII antenna system and referred to as Fr, and one in the far-red (FFr) region (730-740 nm) owing the PSI photosystem.

3. Laser induced chlorophyll fluorescence

The substance emitting the red (Fr) and far-red (FFr) fluorescence of leaves, the red fluorophore, has been identified as Chlorophyll *a*. Although isolated Chlorophyll *b* dissolved in an organic solvent exhibits a red fluorescence, it does not do so *in vivo* because in a leaf the excitation energy is transferred completely to Chlorophyll *a*. At low Chl concentrations, the Fr and FFr increases with increasing Chl concentration (Bushmann, 1981; Stober & Lichtenthaler, 1992; Gitelson et al., 1998; Hák et al., 1990). At higher concentrations, the increase of Chl fluorescence with increasing Chl concentration is mainly detected in the FFr while Fr levels off with rising content. The re-absorption is caused by the overlapping of the short-wavelength range of Chl fluorescence emission spectra with the long-wavelength range of the Chl absorption spectrum. The Fr emission is much more affected by the re-absorption than the FFr, leading to the fluorescence ratio Fr/FFr decrease with increasing Chl content (Gitelson et al., 1998). The simultaneous measure of chlorophyll fluorescence in both red and far-red spectral region allows then the approximate determination of the Chl content of the leaves in a non-destructive way using the ChlF ratios (Hák et al., 1990; Lichtenthaler et al., 1990). In Fig. 4, one illustrates typical chlorophyll emission spectra for *Saccharum officinarum* excited with a blue 2.0 mW LED at 405 nm, for samples under different stages of salinity stress (NaCl concentration).

In a healthy plant (control) the spectrum presents the two distinct emission bands around 685 nm and 735 nm. For plants that experienced very intense stress (200 mM of NaCl), and presenting very low chlorophyll levels, one can clearly see the presence of two additional

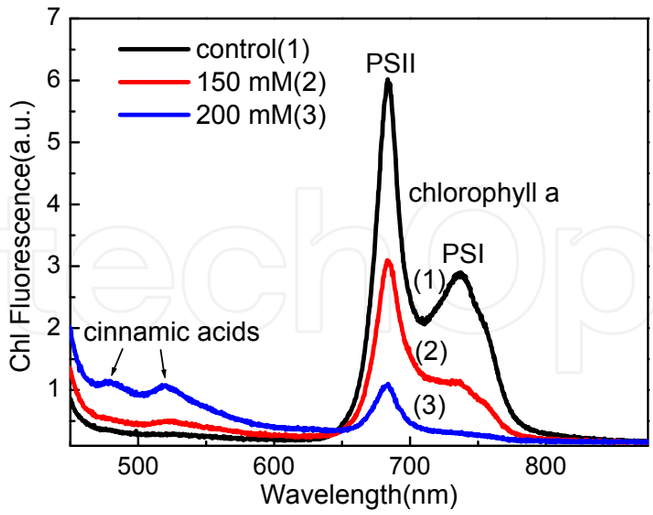


Fig. 4. Emission spectrum of *Saccharum officinarum* plants under intense salinity stress

fluorescence peaks around 440-450 nm and 520-530 nm. In green leaves the blue-green fluorescence is primarily emitted by cinnamic acids (Lichtenthaler & Schweiger, 1998) of the cells walls of the chlorophyll-free epidermis cells. The red and far-red fluorescences, in turn, are emitted by chlorophyll *a* in the chloroplasts of the leaves´ mesophyll cells. We have analyzed the dependence of the chlorophyll fluorescence upon the excitation wavelength and the results revealed that employing either UV (385 nm) or blue (405 nm) excitation light, the red fluorescence around 685 nm is higher when compared to the ones obtained employing blue-green (470 nm), orange (590 nm), and red (627 nm) excitation light, as indeed shown in the spectra of Fig. 5. The re-absorption of the chlorophyll fluorescence on its path towards the leaf surface, leads to different fluorescence spectral shapes for different excitation wavelengths as demonstrated by Agati (Agati, 1998), and Louis and co-workers

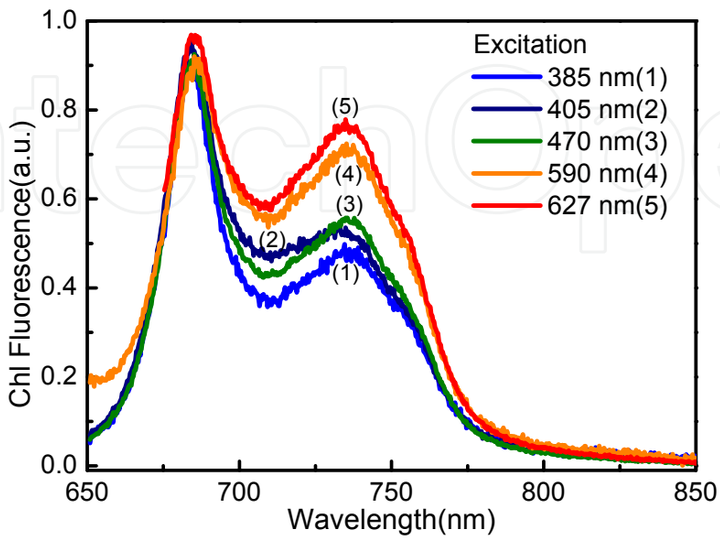


Fig. 5. Emission spectrum of *Saccharum officinarum* at different excitation wavelengths

(Louis et al, 2006) for bean leaves measurements. This is due to the fact that in green leaves, the chlorophylls and carotenoids have a broad absorption band in the 400-500 nm spectral region and blue light does not penetrate very deeply into the leaf tissue, and as a result the fluorescence associated to blue light excitation is mainly generated in the green mesophyll cells close to the leaf`s surface, therefore little absorption occurs. On the other hand, blue-green and orange excitations are not absorbed by carotenoids and penetrates more deeply into the green leaf mesophyll resulting in a chlorophyll fluorescence being generated deeper inside the leaf, from where on its way towards the leaf surface, resulting in a longer pathway and hence the re-absorption is stronger, leading to a less intense red emission compared to the far-red one.

4. Abiotic stress and plants

A number of abiotic stresses impose damage to crop and provokes reduction of yield productivity. Amongst several abiotic stresses, water deficit and soil salinity are the most commonly investigated owing to the extent of cultivated area affected by them. Water scarcity and increase competition for water resources involving several sectors of the production segment (agriculture, industry, hydroelectric energy, etc.) and also for human basic necessities, imposes the study of new concepts of irrigation, in order to adapt the crops to water shortage and maintain satisfactory levels of productivity. On the other hand, salinity affects 7-9 % of the world's land area (Szabolcs, 1994), and the area is increasing (Ghassemi et al., 1995). Nowadays, one of the major technological goals of the energy production is the replacement of the fossil-based fuel for biofuel, mainly due to environmental issues. Bearing that in mind, it is mandatory to investigate the effect of water deficit and salinity stress in plant species with high potential for application in large scale production of nonfossil based fuels.

4.1 Biofuel plants

Brazil is the world's second largest producer of ethanol fuel and the world's largest exporter (Renewable Fuel Association report, 2010). Together, Brazil and the United States lead the industrial production of ethanol fuel, accounting together for 89% of the world's production in 2009. In 2009 Brazil produced 37.7% of the world's total ethanol used as fuel. Brazil is considered to have the world's first sustainable biofuels economy and the biofuel industry leader, a policy model for other countries, and its sugarcane ethanol "*the most successful alternative fuel to date*". Concerning alternative proponents to renewable energy biofuels based on the use of plant oil as a fuel in stationary and mobile engines are the subject of much attention recently. One of the main crops currently being proposed as a diesel/kerosene substitute or extender, is *Jatropha curcas* (Linnaeus) (Openshaw, 2000; Francis et al., 2005) as will be discussed next.

4.1.1 *Saccharum officinarum* (sugarcane)

Sugarcane has been cultivated in Brazil since 1532, and as sugar was one of the first commodities exported to Europe by the Portuguese settlers (Allah n.d.). The first use of sugarcane ethanol as a fuel in Brazil dates back to the late twenties and early thirties of the twentieth century, with the introduction of the automobile in the country. Sugarcane refers to any of 6 to 37 species (depending on which taxonomic system is used) of tall perennial

grasses of the genus *Saccharum* (family Poaceae, tribe Andropogoneae). Native to warm temperate to tropical regions of Asia, they have stout, jointed, fibrous stalks that are rich in sugar, and measure two to six meters tall. All sugarcane species interbreed, and the major commercial cultivars are complex hybrids sugarcane products include table sugar, falernum, molasses, rum, *cachaça* (the national spirit of Brazil), bagasse and mainly ethanol.

4.1.2 *Jatropha curcas* (physicnut)

Jatropha curcas (Linnaeus) is one of the most versatile plants with many attributes and notable potential. It is a small tree belonging to the family of *Euphorbiaceae*. *Jatropha* is easily settled, grows fast and is hardy, and in some way drought tolerant. Thus, it remedies soil degradation, desertification, and deforestation. *Jatropha* is native of tropical America, but now flourish in many parts of the tropics and sub-tropics in Africa/Asia. Various parts of the plant are of medicinal(both human and veterinary purposes) value for instance, and are under intensive scientific investigation. The oil is a strong purgative, widely employed as an antiseptic for cough, skin diseases, and a pain reliever from rheumatism. *Jatropha* latex can heal wounds and also has antimicrobial properties (Openshaw, 2000). Of particular scientific and/or technological interest is that, the fruit of jatropha contains viscous oil that can be used for soap making, in the cosmetic industry, and mainly as a diesel/kerosene substitute or extender (Francis et al., 2005).

5. Material and methods

5.1 Plant material and growing process

Both experiments(water and salinity stress) were conducted in a greenhouse at the Federal Rural University of Pernambuco (UFRPE), in Recife, Brazil, in the period October 2009 to January 2010 for the salinity stress study, and September 2010 to October 2010 for water deficit stress measurements. The seeds, provided by the Center for Technology and Natural Resources (CTRN), Federal University of Campina Grande (UFCG), Brazil, were sown in polyethylene tray containing washed sand as substrate, and samples were watered daily. Following germination, seedlings were irrigated daily in the morning, with nutrient solution containing 742.86 mg L^{-1} soluble fertilizer (Brown Kristalon ®: 3% N, 11% P2O5, K2O 38%, 4% MgO, 11 % S, 0.025% B, 0.004% Mo, 0.01% Cu-EDTA, 0.025% Zn-EDTA, 0.07% Fe-EDTA and 0.04% Mn-EDTA) and 840 mg L^{-1} nitrate Calcium (Viking Ship ® - 15.5% 19.0% N and Ca). This procedure was carried out throughout the whole investigation. After five days of germination, seedlings were selected based upon health and similarity in height and leaf number, and then were transferred to pots made of polyethylene with 10 kg maximum capacity, and containing washed sand substrate. The sand was covered with gravel to prevent soil water evaporation. After 27 days of acclimation, we have established seven treatments defined by the addition of NaCl to the nutrient solution: 0 (control), 25, 50, 75, 100, 150, and 200 mM. The treatment was carried out gradually in order to avoid osmotic shock in the plants. It was conducted by the addition of 25 mM of NaCl per day until the desired salt concentration was attained. The control of salt concentration in the substrate was performed every three days, by measuring the electrical conductivity of the solution drained from the pots. The daily drainage of the solution prevented the accumulation of salts in the substrate. The analysis of chlorophyll a and b content in the leaves was effectuated according to Arnon's methodology (Arnon, 1949). We have performed our experiment in a completely

randomized design, with five replicates per treatment, producing a total of 35 experimental units during the period of 32 days. A randomized design experiment was also carried out for the water stress evaluation of the physicnut plants. We have examined the response through three levels of water stress. The pots were kept at field capacity during 21 days, after which irrigation treatments of drought (nonwatered), medium (50% of water capacity) and slightly below 100% of water field capacity. The 03 treatments were applied during 22 days for sugarcane and 10 days for physicnut plants, in 5 replicates, yielding a total of 15 samples for *Saccarum officinarum*, and 20 for *Jatropha curcas*. The pots were weighted before and after watering and in order to record their mass. The irrigation water contained a balanced nutrient mixture, as the one described in detail in the salinity stress experiment.

In order to evaluate the status of damage caused by the stresses on the plants growing process before visible damage is noticed, we have followed the evolution of the Chl content in the plant leaves using the Fr/FFr chlorophyll fluorescence ratio. The absolute emission signal of leaves can vary from sample to sample due to small differences such as excitation and sensing angles of the fluorescence, and the roughness and scattering properties of the leaf surface. Thus, the absolute fluorescence usually varies to a large extent than the fluorescence ratio. The fluorescence ratio turns out to produce much lower variations from leaf to leaf, resulting in a reliable and reproducible method for the quantification of changes in the fluorescence characteristics of leaves.

5.2 Experimental

In the experiments, the chlorophyll fluorescence was measured under steady-state conditions, in 20 min predarkened intact leaves, and we have employed as the excitation source, a blue LED at 405 nm with 10 nm of bandwidth and delivering a maximum power of 2.2 mW. The choice relies upon the fact that its wavelength resides within the main absorption band of Chl *a*, producing much higher fluorescence emission intensity. Red and far-red chlorophyll fluorescence emission around 685 nm and 735 nm, respectively, were observed and analyzed as a function of the stress intensity (NaCl concentration and amount of irrigation water). The LICF experiments were carried out within a time interval of 32 and 22 days (sugarcane) for the salinity and water stress evaluation, and 10 days for both sudies in physicnut. The measurements were performed every 4 days in order to monitor the evolution of the ChlF ratio during the NaCl treatment of plants. For the water stress a 2 days time intervals was utilized between measurements. The Fr/FFr ratio was evaluated using Gaussian shaped fluorescence fitting curves and analyzed as a function of time, and salinity intensity and water irrigation amount. Excitation and sensing were performed on the adaxial leaf surface. The ChlF experimental apparatus consisted of a fiber integrated LED source, spectrometer and light detector (Ocean Optics USB2000). The detection system had an overall operating spectral resolution of ~ 1.0 nm. The excitation source was directed to the leaf surface by means of a 200 μm diameter fiber cable which possessed a mechanical system at the fiber cable output extremity in order to prevent any ambient light of reaching the leaf surface during the measurements. Moreover, as the fiber itself was in contact with the leaf surface, it effectively shadowed away any leakage of ambient light. All spectra presented in this study were handled employing appropriate (Ocean Optics-SpectraSuite) software of the spectrometer. The data was stored and analyzed in a personal computer using a commercially available software (Origin 6.0).

6. Results and discussion

6.1 Salinity stress

Amongst abiotic stresses, salt stress is known to disturb the normal physiological processes and chloroplast ultrastructure at various levels (Allakhverdiev & Murata, 2008; Hasegawa et al., 2000; Munns, 2002; Sayed, 2003). The extent of the disturbance by NaCl ions depends upon the concentration and the plant tolerance. The decline in productivity observed in several plant species under salt distress is commonly associated with reduction in the photosynthetic capacity. Although the factors that limit photosynthesis in salt stressed plants are unclear, the effect of salinity stress on a number of species is quite evident and have been investigated in the past few years by several research groups worldwide (Yamane et al., 2003; Liu & Shen, 2006; Jimenez et al., 1997; Meloni et al., 2003; Lin et al., 2007; Zribi et al., 2009; Mehta et al., 2010).

6.1.1 *Saccharum officinarum*

In this experiment we have followed the time evolution and the shape of the ChlF spectral profiles during a 35 days period of time, evaluating the effect of soil salinity in sugarcane plants. The dependence of the ChlF ratio upon the NaCl concentration was carried out for two varieties of *Saccharum officinarum* and the results are depicted in Fig. 6a (RB863129) and 6b (RB867515). The results clearly show an evident decrease in the chlorophyll content corresponding to a increase in the Fr/FFr ratio of the leaves experiencing an intense salinity stress, while the control sample undergo a steady increase in the chlorophyll concentration as time evolves. The results also indicates that, in the early stage (2-3 weeks) of the NaCl treatment, both plants follow the expected increase in the Chl content. After the 3rd week, however, a competition between the normal chlorophyll concentration evolution and the counter effect of the salinity distress takes place, and plants start to debilitate rapidly with time. The missing data at the fifth week in the graph of Fig. 6a (RB863129 variety) is because the samples did not resist to the salinity distress and samples remaining in the experiment would not provide reliable data for the Fr/FFr ratio.

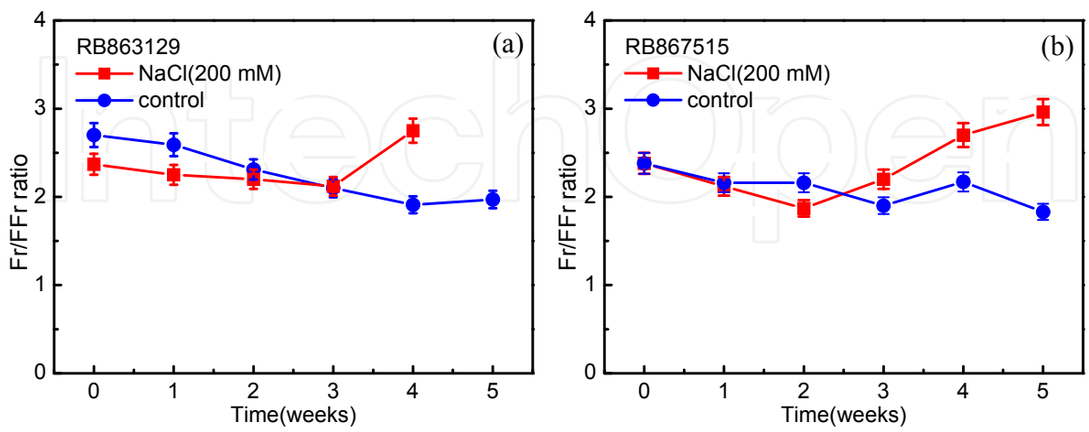


Fig. 6. Chlorophyll fluorescence ratio as a function of time

The chlorophyll fluorescence spectrum during the induction kinetic, the so called Kautsky effect, have been also investigated. When a 20 min pre-darkened plant leaf is submitted to

excitation light, the onset of the photosynthetic process can be analyzed through the decrease of the Chl fluorescence from the initial maximum reached in the first second to the stead-state value after a few minutes of illumination. During the induction kinetics the wavelength position of the Chl fluorescence does not change, but the two emission bands decline at different time rates, leading to a decrease in the Fr/FFr ratio as can be observed in the results depicted in Fig. 7.

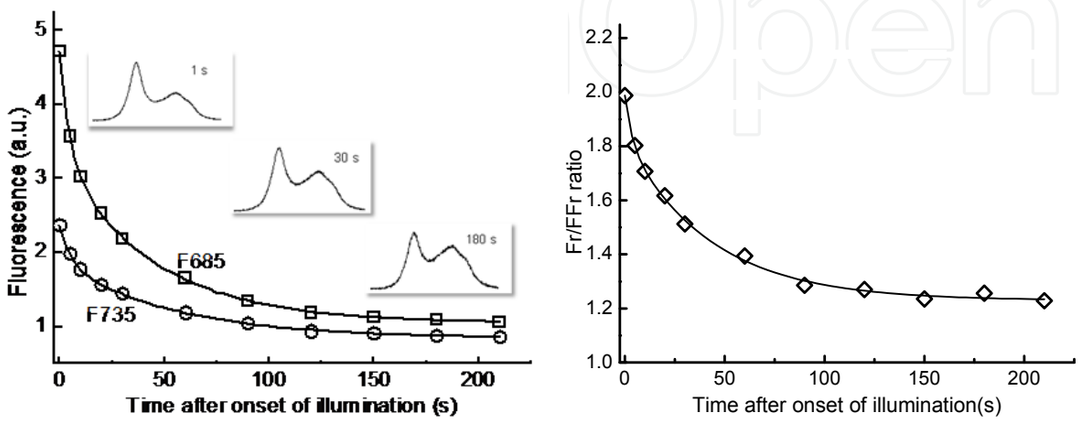


Fig. 7. Chlorophyll fluorescence ratio as a function time after onset of illumination

The dependence of the Chl fluorescence ratio upon the NaCl concentration was carried out and the results are depicted in Fig. 8. The results indicate that the salinity plays a very important role in the chlorophyll concentration of leaves tissues in both plants spieces, with a significant reduction in the Chl content for NaCl concentrations in the 70 - 100 mM range, where the fluorescence ratio curve exhibits a noticeable decrease for 100 mM NaCl concentration, which is a clear indication of Chl content decrease. This is corroborated by the spectrophotometric analysis presented in the same graph, which determines the chlorophyll content through *in vitro* absorption spectrum of leaves pigments in acetone extract.

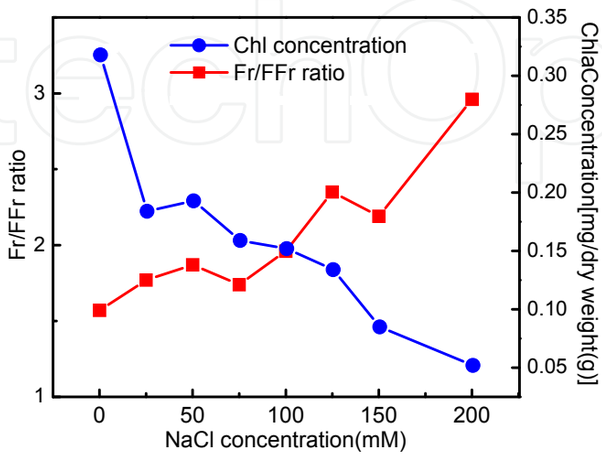


Fig. 8. ChlF ratio (left) and Chl *a* content (right) versus NaCl concentration

6.1.2 *Jatropha curcas*

The spectra shown in Fig. 9 are associated with *Jatropha curcas* plants treated with the maximum NaCl concentration of 200 mM at three different stages of the salinity stress time evolution. In the first day of experimentation, both the healthy plant (control) and the plants under high salt concentration presented spectra showing the two distinct emission bands around 685 nm and 735 nm. After 16 days of treatment(not shown in Fig. 9), on the other hand, the samples under intense stress exhibited a distinct reduction in the chlorophyll content as demonstrated by the noticeable increase in the Fr/FFr fluorescence ratio. The control sample, however, showed a significant Fr/FFr ratio reduction owing to the increase in the Chl content of the leaves.

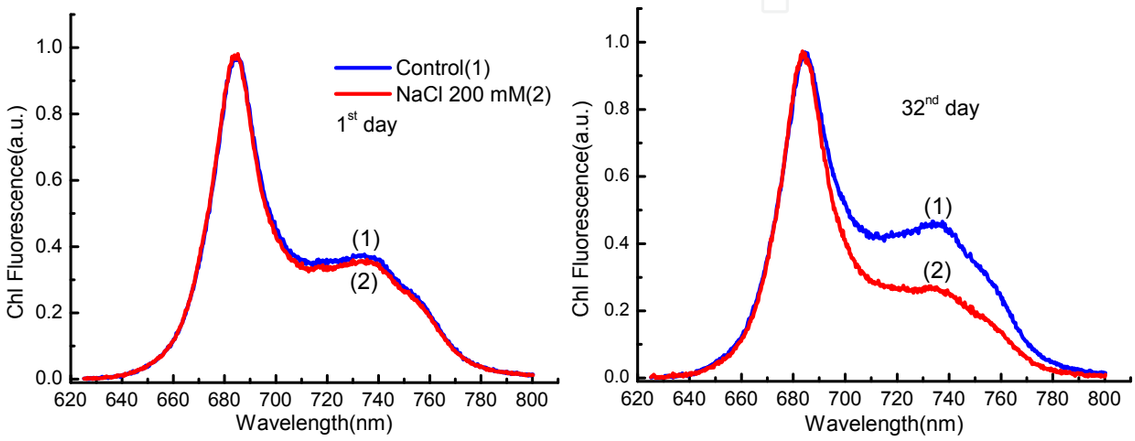


Fig. 9. Typical chlorophyll emission spectra of *Jatropha curcas* plants excited at 405 nm

The ChlF ratio time evolution for a 32 days period of time and several stress intensities (NaCl concentration) was studied and the results are depicted in Fig. 10.

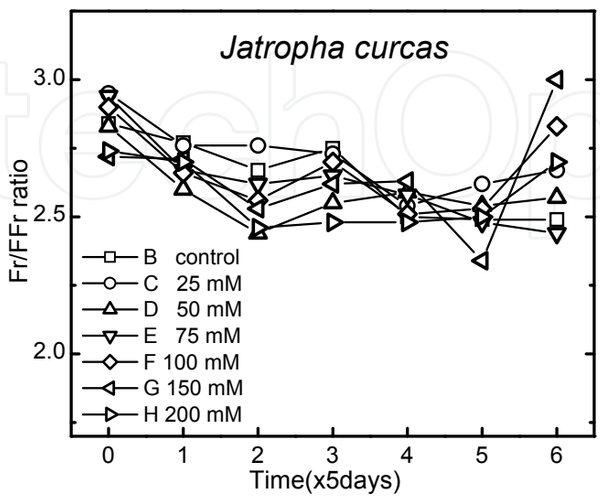


Fig. 10. Chlorophyll fluorescence ratio as a function of time

The results clearly show that there exists an inicial decrease in the ratio during the first few days of salt stress indicating increase in the chlorophyll content of the leaves submitted to salinity stress. This behavior observed for all stressed plants is attributed to the reaction of the plants to minimize the effect of distress caused by the high salinity of the soil. As time evolves, one observes that all samples under stress experience a steady behavior with the Fr/FFr ratio presenting basically the same value up to the 22nd day of stress. The control sample however, undergo a steady increase in the chlorophyll concentration as time evolves reaching the maximum value 24 days after the measurements had commenced. Following the 3rd week, however, a competition between the normal chlorophyll concentration evolution and the counter effect of the salinity distress takes place, and plants start to debilitate with time. It is important to point out that the salinity stress provokes a minor effect in the chlorophyll a content of *Jatropha curcas* leaves for NaCl concentrations up to 100 mM. This salinity distress resistance of *Jatropha curcas* indicates that this species can be considered as a main alternative crop for biofuel production in high salinity soil regions.

In order to demonstrate in detail the effect of the soil salinity on the *Jatropha* plants, it is presented in Fig. 11, the time evolution of the stress in the control and the plant under extreme distress (200 mM), and results clearly show that high salinity provokes detectable damage in the plants only after 20 days of stress exposure. In order to evaluate the effect of the salt stress on the chlorophyll content of leaves, we have carried out measurements at the end of the experimentation period (dismount), and the dependence of the Chl content, using the nondestructive fluorescence ratio and the conventional technique upon the NaCl concentration, was examined and the results are depicted in Figure 11. The results follow the trend presented in the time evolution of the salinity distress imposed to the plants and as such, the salinity plays a minor role in the chlorophyll concentration of leaves tissues. This is corroborated by the spectrophotometric analysis presented in the same graph. The chlorophyll content do not vary substantially for concentrations in the 25 to 200 mM, presenting a variation of less than 10 % of the initial value for the stressed plants.

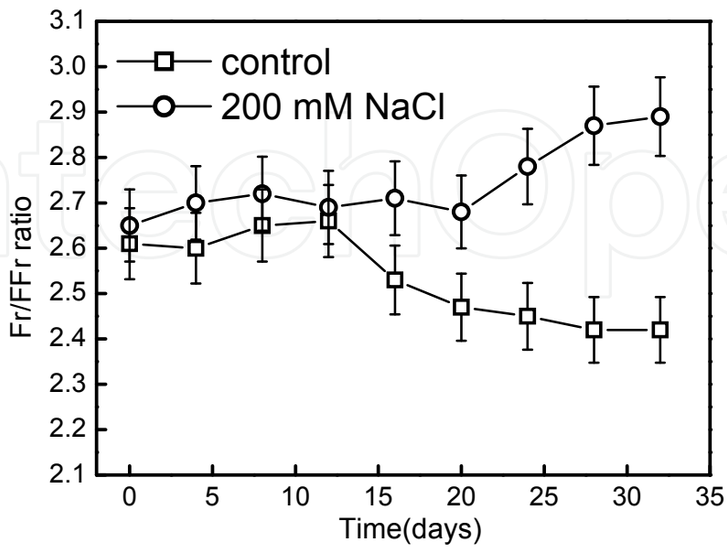


Fig. 11. Time evolution of Fr/FFr ratio for control and plant under extreme salt stress

The dependence of the Chl content, using the nondestructive fluorescence ratio and the conventional technique upon the NaCl concentration, was carried out and the results are depicted in Figure 12. The results follow the trend presented in the time evolution of the salinity distress imposed to the plants and as such, the salinity plays a minor role in the chlorophyll concentration of leaves tissues. This is corroborated by the spectrophotometric analysis presented in the same graph. The chlorophyll content do not vary substantially for concentrations in the 25 to 200 mM, presenting a variation of less than 10 % of the initial value for the stressed plants.

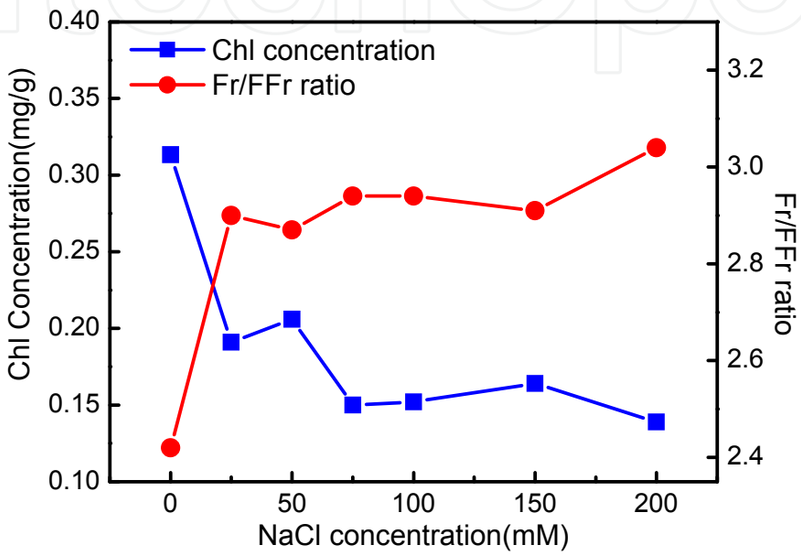


Fig. 12. ChlF ratio and Chl concentration as a function of NaCl concentration

6.2 Water stress

Nowadays, one of the major technological goals of the energy production, is the replacement of the fossil-based fuel for biofuel, mainly due to environmental issues. Bearing these concepts in mind, it is imperative to study the effects of water deficit in plant species with high potential for application in mass production of nonfossil based fuels. One of the main crops currently being proposed as a diesel/kerosene substitute or extender, is *Jatropha curcas* (Linnaeus) (Openshaw 2000, Francis et al., 2005). Water stress studies have been already carried out in several plant species, seeking for responses of different mechanisms in leaves under water distress (Theisen, 1988; Chappelle et al., 1984; Dahn et al., 1992; Broglia, 1993; Munns, 2002; Marcassa et al., 2006; Abou Kheira & Atta, 2009; Maes et al., 2009; Caires et al., 2010; Robredo et al., 2010; Patane & Cosentini, 2010; Tushar et al., 2010; Silva et al., 2010). In this section, the effect of water deficit in jatropha plants is investigated using chlorophyll fluorescence spectroscopy. To this end, we have investigated the response of *Jatropha* plants to water stress within three levels of water deficit. Fig. 13 shows the evolution of ChlF spectral profile of the samples under maximum water stress (nonwatered plants) within a 10 day time interval. As can be observed from data, in the very begining of the experiment both control and nonwatered samples present basically the same ChlF spectral profile. In the fifth day of investigation one observes a discrete change in the

spectral profile with the control sample presenting a lower Fr/FFr ratio while the stressed ones maintain the initial profile. As time evolves, the stressed sample presents a more pronounced spectrum with the fluorescence ratio decreasing even further and the control sample exhibiting basically the same ChlF ratio and spectral profile.

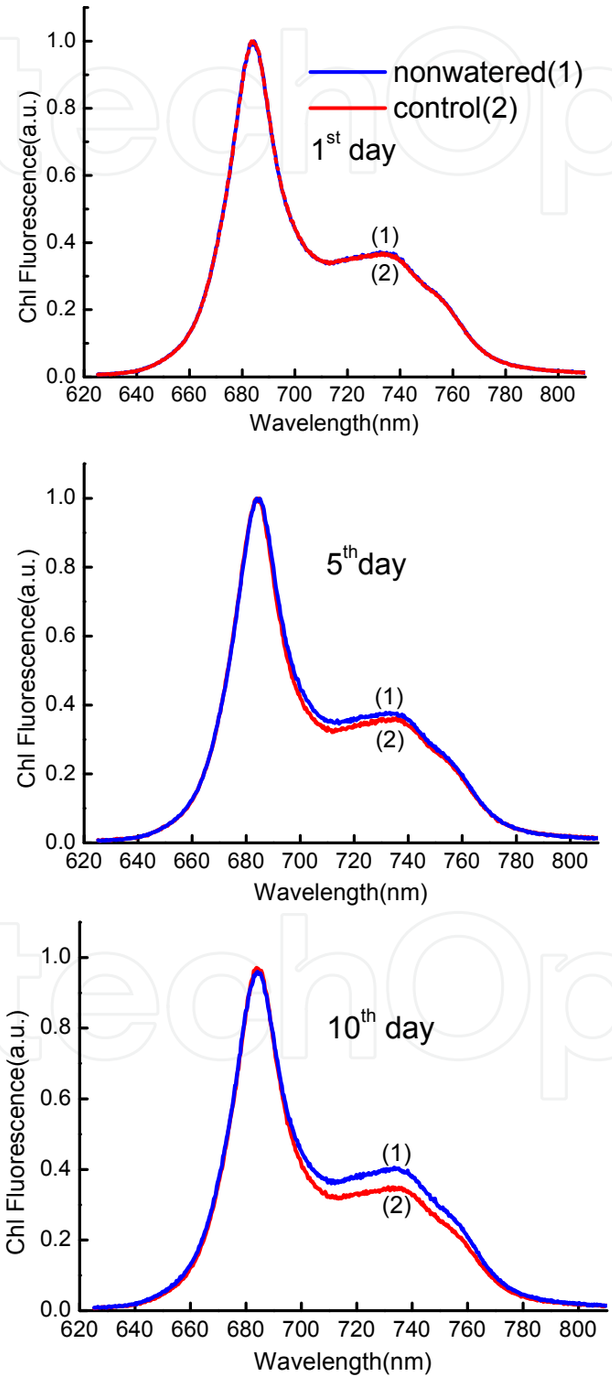


Fig. 13. Typical chlorophyll fluorescence spectrum of *Jatropha curcas* samples

This behavior of the *Jatropha curcas* under water stress is better visualized examining the time evolution of the ChlF ratio, as depicted in graph of Fig. 14. The data illustrated in Fig. 14, show a very unusual behavior with a decrease of the Fr/FFr ratio for the samples under maximum water stress as time evolves in the water distress case. This is to be compared with the behavior of the plants undergoing salinity stress, which exhibits an opposite tendency. The ChlF ratio decreases by approximately 18% within the first 10 days of the experiment for samples under highly intense water stress. It is also important to point out that the samples under mild stress (50% field capacity) did not undergo detectable changes either visual or in the Fr/FFr ratio along the 10 days period. These results would indicate, in principle, that the chlorophyll content of the highly stressed samples are increasing as the time evolves, while the control and mildly stressed samples maintained their initial concentrations. Nevertheless, the Chl concentrations obtained using conventional spectrophotometric techniques based upon Arnon's method (Arnon, 1949) showed no appreciable variation in the Chl concentration for all samples. The measured concentrations were 1.5 mg/g, 1.52 mg/g, and 1.53 mg/g for the control, 50% field capacity, and 0%(nonwaterd) field capacity, respectively. The decrease of the Fr/FFr ratio was observed previously by Chappelle and co-workers in soybeans (Chappelle et al., 1984), Dahn and co-workers in maize (Dahn et al., 1992), and by Marcassa and co-workers in orange trees (Marcassa et al., 2006). The most visible sign of water stress in the majority of plants is wilting. But, in our observations this process was not evident. The ChlF spectral profile, however, presented clearly detectable changes, particularly in the ChlF ratio. One possible reason for that is the efficiency of photosynthesis appears to be impaired. Another possible reason is that the quenching effect of water upon chlorophyll fluorescence is reduced due to the decrease in leaf water (Chappelle et al., 1984).

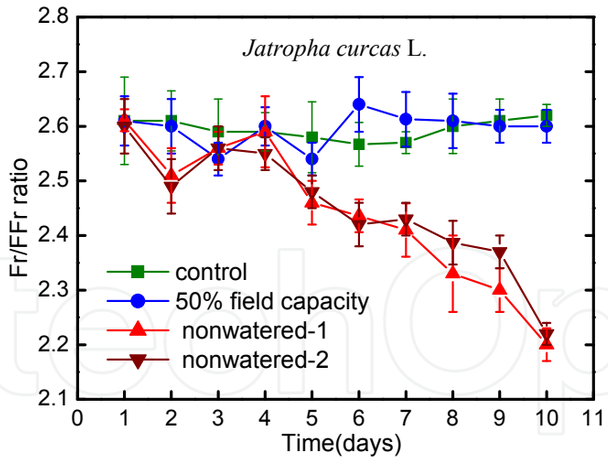


Fig. 14. Fluorescence ratio time evolution for *Jatropha curcas* under water stress

We have also examined the water deficit stress on *Saccharum officinarum* plants and the results exhibited a similar behavior as the *Jatropha curcas* plants, with the Fr/FFr ratio decreasing with stress intensity and time. It was also possible to detect the stress in the early stages and prior to visual inspection, as can be inferred from graph depicted in Fig. 15. The results for the sugarcane samples corroborates the behavior shown in our measurements with *Jatropha curcas* and the ones reported elsewhere (Chappelle et al., 1984; Dahn et al., 1992; Broglia, 1993).

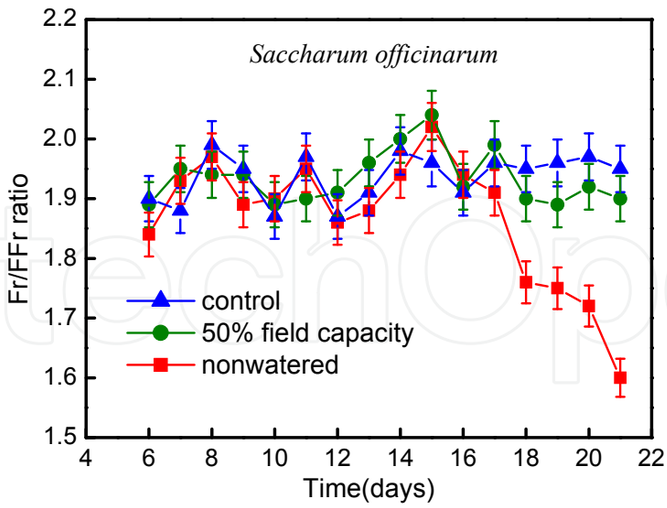


Fig. 15. Fluorescence ratio time evolution for *Saccharum officinarum* under water stress

7. Conclusion

Light-emitting-diode induced chlorophyll fluorescence analysis was employed to investigate the effect of water deficit and salt stress upon the growth process of *in vivo* leaves tissues of Brazilian biofuel plants species. The chlorophyll fluorescence emission spectra of 20 min predarkened intact leaves were studied employing several excitation wavelengths in the UV-VIS spectral region. We have chosen *Saccharum officinarum* and *Jatropha curcas* L. plants owing to their application in large scale industrial production of biofuel. Red and far-red chlorophyll fluorescence emission signals around 685 nm and 735 nm, respectively, were examined as a function of the stress intensity, and time. The chlorophyll fluorescence data indicated that the soil salinity plays a major hole in the chlorophyll concentration of *Saccharum officinarum* leaves, with a significant reduction in the Chl content for NaCl concentrations of a few tens of miliMolar. On the other hand, concerning *Jatropha curcas* plants, the soil salinity plays a minor role in the chlorophyll concentration of leaves tissues for NaCl concentrations in the 25 to 200 mM range, and in both cases, results agreed quite well with those obtained using conventional destructive spectrophotometric methods. The technique was also employed to investigate the effect of water deficit on the growing process of the biofuel plants species. The Chl fluorescence ratio analysis permitted detection of damage caused by water deficit in the early stages of the plants growing process with a significant variation of the Fr/FFr ratio as compared to the control sample in the first 10 days of the plant growing process. The results suggested that the technique can potentially be used as an early-warning indicator of stress caused by water deficit. It is also important to emphasize that salinity stress produced a minor effect in the chlorophyll content of *Jatropha curcas* leaves for NaCl concentrations up to 100 mM. The resistance of *Jatropha curcas* to salinity distress indicates that this species is a viable alternative crop for biofuel production in high salinity soil regions. The technique has also been applied to detect and monitor early stages of distress caused by heavy metal (Cd, Pb, Ni, Zn, etc) soil contamination (Gopal et al, 2002; Maurya et al, 2008; Ventrella et al, 2009)

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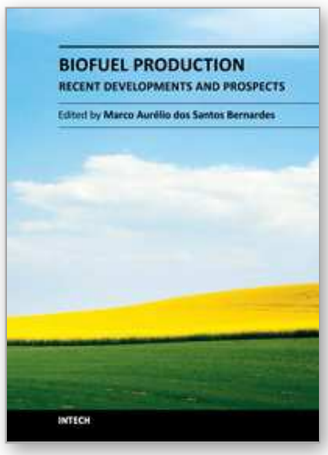

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This book aspires to be a comprehensive summary of current biofuels issues and thereby contribute to the understanding of this important topic. Readers will find themes including biofuels development efforts, their implications for the food industry, current and future biofuels crops, the successful Brazilian ethanol program, insights of the first, second, third and fourth biofuel generations, advanced biofuel production techniques, related waste treatment, emissions and environmental impacts, water consumption, produced allergens and toxins. Additionally, the biofuel policy discussion is expected to be continuing in the foreseeable future and the reading of the biofuels features dealt with in this book, are recommended for anyone interested in understanding this diverse and developing theme.

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