J Fluoresc (2012) 22:623–630
DOI 10.1007/s10895-011-0998-9

ORIGINAL PAPER

# Water Deficit and Salt Stress Diagnosis Through LED Induced Chlorophyll Fluorescence Analysis in *Jatropha curcas* L.

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Received: 30 May 2011 / Accepted: 14 October 2011 / Published online: 4 November 2011


**Abstract** LED induced chlorophyll fluorescence analysis is employed to investigate the effect of water deficit and salt stress upon the growth process of *Jatropha curcas* L.. Red(Fr) and far-red(FFr) chlorophyll fluorescence around 685 nm and 735 nm, respectively, were observed and examined as a function of the stress intensity(salt concentration and water deficit). The fluorescence ratio Fr/FFr which is a valuable nondestructive and nonintrusive indicator of the chlorophyll content of leaves was exploited to monitor the *jatropha* plants under stress. The data indicated that salinity plays a minor role in the chlorophyll concentration of leaves for NaCl concentrations in the 25 to 200 mM range. The fluorescence ratio also permitted the detection of damage caused by water deficit in the early stages of the plants growing process. A significant variation of the Fr/FFr ratio was observed in the first 10 days of the experiment, and before signs of visual stress became apparent. The results suggest that the Fr/FFr ratio is an early-warning indicator of water deficit stress

**Keywords** Fluorescence · Chlorophyll · Abiotic-stress · Jatropha curcas · Biofuel · Water · Salt

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## Introduction

*Jatropha curcas* (Linnaeus) is a multipurpose plant with many attributes and notable potential. *Jatropha* is easily settled, grows fast and is hardy, and in some way drought tolerant. Thus, it remedy 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 [29]. 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 [12]. Thus, it is imperative to study the effect of abiotic stresses in such versatile plant using new noninvasive and nondestructive remote sensing diagnostic techniques. In the past few years there has been a widespread scientific and technological interest in laser remote-sensing techniques to investigate the status of terrestrial vegetation [36]. These techniques exploit the fluorescence emission from the plant leaves generated in the photosynthesis process. Photosynthesis is a biophotonic

mechanism by which green plants exploit solar energy to reduce $CO_2$ and oxidize $H_2O$. 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 [4]. Photosynthesis occurs in the chloroplasts where the photosynthetic pigments reside. Chlorophyll molecules are 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 indicate the wavelengths corresponding to the maxima of the absorption spectra of the two species of Chlorophyll a. A small part of the absorbed light energy 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 around 680–690 nm and far-red 730–740 nm spectral regions and is termed as Chlorophyll Fluorescence(ChlF) [6, 33]. 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(LICF) spectroscopy remote-sensing has drawn much attention recently owing to the nonintrusive and nondestructive nature of the technique [10, 17, 36]. The technique can be applied for Chl determination in basic photosynthesis research, agriculture, horticulture, and forestry. In this work, LED light induced Chl fluorescence is employed to evaluate the effect of abiotic stresses(water deficit and soil salinity) upon the evolution and characteristics of in vivo chlorophyll emission spectra of leaves of brazilian physicnut oil plant for biofuel.

## Materials and Methods

### Plants

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 5 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 3 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 [5]. 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 in 5 replicates, yielding a total of 15 samples. 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.

### LICF(LED 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 emissions increase with increasing Chl concentration [8, 13, 14, 35]. At higher concentrations, the increase of Chl

fluorescence with increasing Chl concentration is mainly detected in the FFr signal while Fr emission levels off with rising Chl 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. As demonstrated by Lichtenthaler and co-workers [18], 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. 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 ratio [2, 19]. 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. In our experiment, 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. We have investigated the dependence of the chlorophyll fluorescence upon the excitation wavelength and the results revealed that employing either UV(380 nm) or blue(405 nm) excitation light, the emission intensity is much higher when compared to the the signals obtained employing blue-green(470 nm), orange(590 nm), or red (627 nm) excitation light. The re-absorption of the chlorophyll fluorescence on its path towards the leaf surface, leads to different emission spectral profiles for different excitation wavelengths as demonstrated by Agati [2], and Louis et al. [22] 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. For the measurements herein reported, we have utilized a blue excitation source at 405 nm, in order to obtain a maximum absorption, resulting in maximum fluorescence emission.

The choice relies upon the fact that its wavelength resides within to the maximum of the absorption spectrum of Chl a, producing much higher Chl a 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, for the salinity and water stress evaluation, respectively. 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 interval was utilized between measurements. The Fr/FFr ratio was evaluated using Gaussian shaped fluorescence fitting curves and analyzed as a function of the salinity and water irrigation amount. Excitation and sensing were performed on the adaxial leaf surface. The 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 communication 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).

### Chlorophyll Analysis

At the end of the LICF experiments the remaining leaves of all plants were used for pigment sampling. The chlorophyll contents were determined in 80% acetone extract on a Biospectro UV-SP-220 spectrophotometer using equations of Arnon [5] for calculations, and the results used to compare with the ones obtained by the ChlF ratio technique.

## Results and Discussion

### Salt Stress

Amongst abiotic stresses, salt stress is known to disturb the normal physiological processes and chloroplast ultrastructure at various levels [3, 15, 28, 32]. The extent of the disturbance by NaCl 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

have been investigated recently using several diagnostic techniques [16, 20, 21, 25, 26, 39–41]. In order to evaluate the status of damage caused by the salinity on the *jatropha* 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, for different NaCl concentrations and during a period of time of 32 days with measurents carried out in 4 days intervals.

In Fig. 1, one presents typical chlorophyll emission spectra of *Jatropha curcas* leaves. When excited with either UV or blue radiation, plant leaves may exhibit a fluorescence emission spectrum in two regions blue-green(400–550 nm) and red-far-red region(650–800 nm). However, in our case the fluorescence emission intensity in the blue-green spectral region was too small to be used as reliable chlorophyll fluorescence signatures, and was detected only in extreme cases with plants presenting 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 atributted to the PSII antenna system, and one in the far-red(FFr) region (730–740 nm) owing the PSI photosystem. The spectra shown in Fig. 1 are associated with 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, 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. For plants presenting very low chlorophyll levels, it is observed the presence of two additional fluorescence peaks around 440–450 nm and 520–530 nm(not shown in spectra of Fig. 1). In green leaves the blue-green fluorescence is primarily emitted by cinnamic acids [19] of the cells walls of the chlorophyll-free epiderms cells. The red and far-red fluorescences, in turn, are emitted by chlorophyll a in the chloroplasts of the leaves' mesophyll cells. In the 32nd day of treatment the difference in the spectral profile for both samples is quite evident with the Fr/FFr ratio increasing even further for the stressed plant.

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. 2. 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

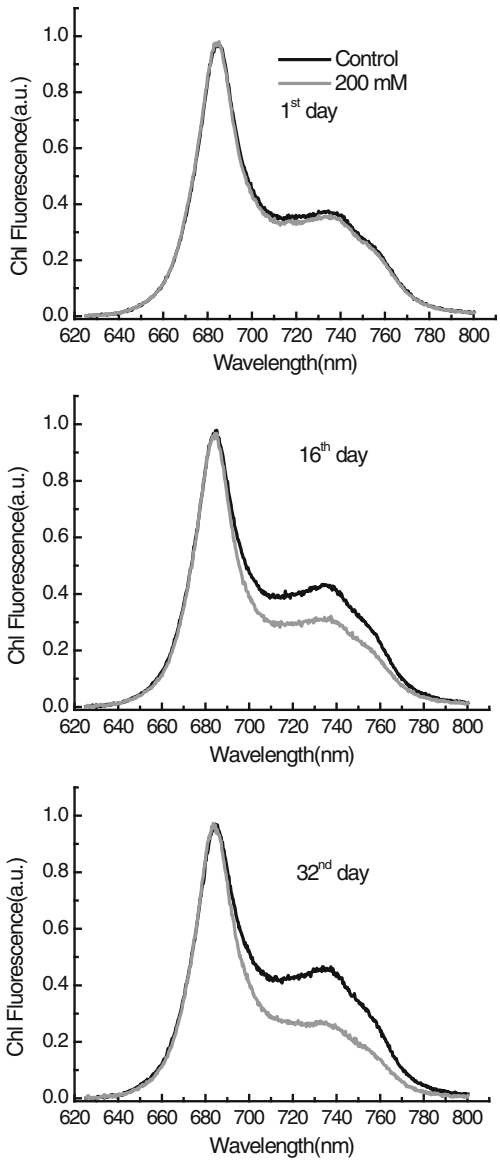


**Fig. 1** Spectral profile time evolution of *Jatropha curcas* samples and under salt stress

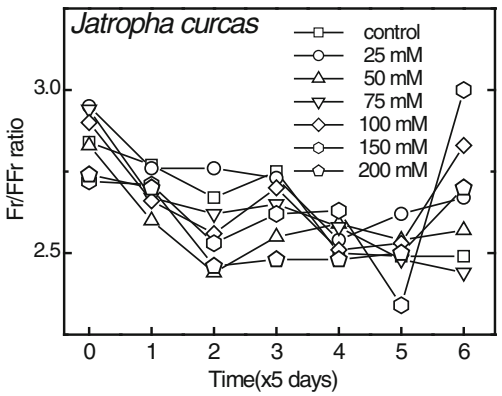


**Fig. 2** Chlorophyll fluorescence intensity ratio as a function of time for different salinity stress conditions (NaCl concentration)

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 provoques 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. 3, 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 Fig. 4. 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.

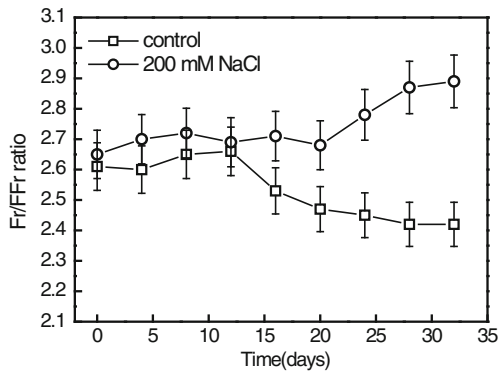


**Fig. 3** Time evolution of the fluorescence ratio for control and plant under extreme salt stress(200 mM)

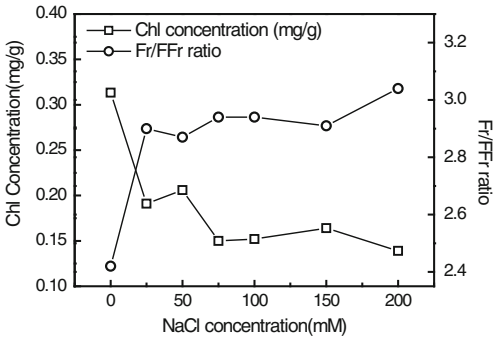


**Fig. 4** Chlorophyll concentration as a function of salt stress intensityusing Fr/FFr ratio(left) and Arnon's methodology(right)

## Water Stress

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. 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) [12, 29]. Water stress studies have been already carried out in several plant species, seeking for responses of different mechanisms in leaves under water distress [1, 7, 9–11, 23, 24, 28, 30, 31, 34, 37, 38]. 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. Figure 5 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 one maintain its 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. 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. 6.

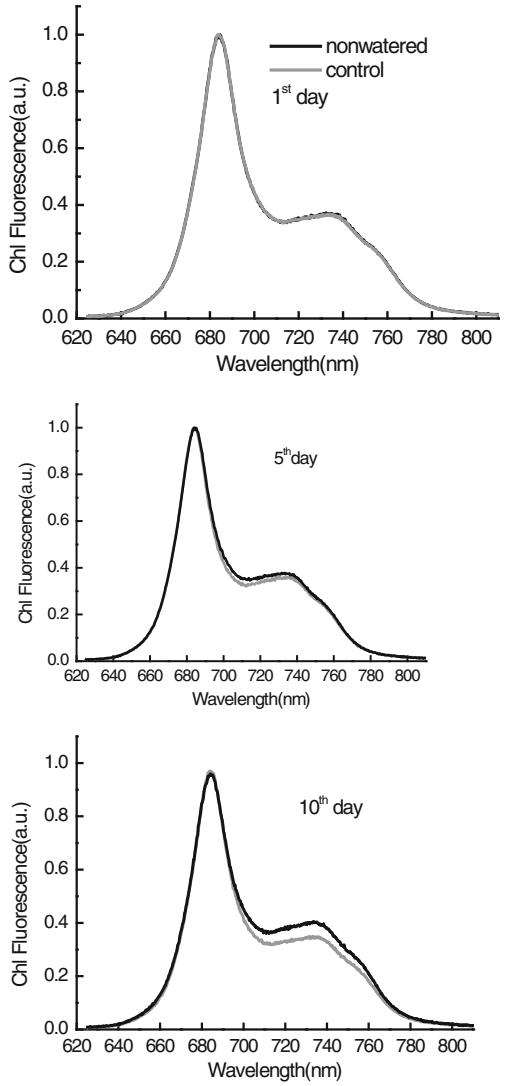


**Fig. 5** Spectral profile time evolution of *Jatropha curcas* samples under water stress

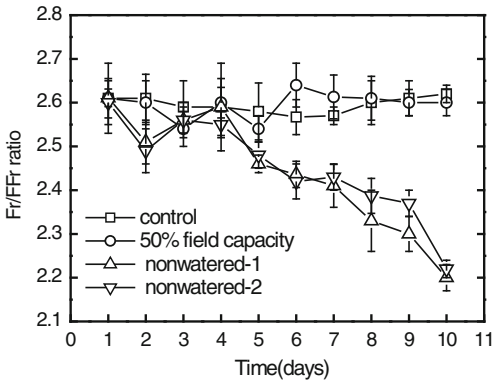


**Fig. 6** Chlorophyll fluorescence ratio as a function of time for different levels of water stress

The results presented in Fig. 6, 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 [5] 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 [10], Dahn and co-workers in maize [11], and by Marcassa and co-workers in orange trees [24]. 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 [10]. It is very important to point out that *Jatropha curcas* has shown to possess an inbuilt capacity to grow under severe water deficit conditions and also to exhibit high resistance to salinity distress, indicating that this species is a viable alternative crop for biofuel production under severe stress conditions.

## Conclusions

Light-emitting-diode induced chlorophyll fluorescence analysis was employed to investigate the effect and time evolution of *Jatropha* plants under water deficit and salt stress. Red and far-red chlorophyll fluorescence emission signals around 685 nm and 735 nm, respectively, were examined as a function of the salinity concentration, irrigation availability, and time. The ChlF technique data indicated that the salinity plays a minor role in the chlorophyll concentration of leaves tissues for NaCl concentrations in the 25 to 200 mM range, and results agreed quite well with those obtained using conventional destructive spectrophotometric methods. The Chl fluorescence ratio analysis also 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. The technique has also been applied to detect early stages of distress cause by heavy metal soil contamination [27]. It is also important to emphasize that salinity stress provoqued a minor effect in the chlorophyll content *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.

**Acknowledgements** The financial support for this research by CNPq-Conselho Nacional de Desenvolvimento Científico e Tecnológico (Proc.472328/2008-05) and FACEPE(Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco) APQ 0504–1.05/08, Instituto Nacional de Ciência e Tecnologia(INFOS) is gratefully acknowledged. Elias A. da Silva Jr. is supported by a graduate studentship from FACEPE. Mr. Ronaldo Alves is supported by an undergraduate studentship from the PIBIC/UFRPE.

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