DETECTION OF CHANGE IN CHLOROPHYLL FLUORESCENCE USING LOW SPECTRAL RESOLUTION SPECTROMETER- A STUDY FOR TEMPERATURE INDUCED STRESS DETECTION

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March, 2017

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Enschede, The Netherlands, [March, 2017]

Thesis submitted to the Faculty of Geo-Information Science and Earth Observation of the University of Twente in partial fulfilment of the requirements for the degree of Master of Science in Geo-information Science and Earth Observation.
Specialization: Water Resources and Environmental Management

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ABSTRACT

To increase the agricultural production, pre-symptomatic detection of plant stress is necessary as stress affects the growth and photosynthesis of the crop plant. Over the last two decades, several scientific studies have shown that the steady state chlorophyll fluorescence (ChlF) could be used as a probe to the plant stress and plant physiological conditions. To use ChlF as an indicator of plant stress, detection and quantification of ChlF are necessary. Most studies investigating the ChlF for stress detection make use of hyper-spectral spectrometer to detect the ChlF. While using hyper-spectral spectrometer, instrument need to have a very high spectral resolution (<1 nm) and its often need to couple with an integrating sphere and high/low pass filters. These technical requirements of instrumentation makes detection of ChlF expensive, difficult and limits the use of low spectral resolution spectrometer.

In this study, we detected the spectral change in ChlF using low spectral resolution spectrometer (> 1 nm) (without using an integrating sphere or short/long-pass filters) and investigated the effect of high temperature stress on transient change in ChlF on the illumination of dark adapted plant. The ChlF was detected by using two approaches, in the first approach- the plant leaf was illuminated with the full spectrum of photosynthetically active radiation (PAR) and reflected radiance from leaf was recorded at two physiological stages where ChlF varied significantly (variation in light intensity and sudden illumination of the dark adapted plant). Presence of ChlF peak (at 685 nm and 740 nm) was then observed in the difference spectrum of reflected radiances. In the second approach, the plant leaf was illuminated with the ChlF excitation light whose spectrum does not overlap with ChlF emission wavelengths. The reflected radiance was acquired and it was observed for presence of ChlF signature. The high temperature stress was simulated in laboratory and both the approaches were tested for their potential to detect the effect of temperature stress on change in ChlF.

The study was conducted over C_3 (plant that uses C_3 carbon fixation pathway) and C_4 (plant that uses C_4 carbon fixation pathway) plants because of their different mechanism to adapt temperature stress, which was possible to study in this research. Spectral measurements were performed using ASD FieldSpec Pro FR spectroradiometer (ASD; spectral resolution 3 nm). The ChlF excitation sources used were: halogen light and LED light (light consisting blue and green LED of wavelength 460 nm and 660 nm respectively).

Experiments' results show that the spectral measurement using ASD can track small changes in ChlF excited by halogen and LED light, provided that the signal to noise ratio of the recorded ASD signal is high and ChlF excitation source produces stable light output. The measurements using ASD detect the effect of temperature stress on transient change in ChlF and show that the high temperature stress causes significant fluctuations in functioning of the plant, giving rise to variations in reflectance and ChlF. Results show that the high temperature stress makes it difficult to adjust steady state ChlF efficiently for C_3 and C_4 plant. The examination of light source show that the halogen light is not suitable for ChlF studies as it produces heat stress to the plant while the LED light could be used efficiently.

Here we recommend to extend this study to further investigate the use of low spectral resolution spectrometers to detect the change in ChlF at canopy level using blue LED light as a ChlF excitation source.

Keywords

Chlorophyll fluorescence, spectral resolution, temperature stress, vegetation stress

ACKNOWLEDGEMENTS

This thesis would have not successfully completed without the help and the support from many of you,

Firstly I thank the Sai, the one who rooted wisdom, strength, love and health in to me. He gave power to believe in myself and to struggle all the way.

I thank the EP-NUFIC for providing me an opportunity to pursue this education, without your financial support my study was not possible.

I thank to the department of Water Resources and Environmental Management of the Faculty of Geo-information Science and Earth observation, University of Twente for outstanding education, support and facilities that I have received here. You created valuable learning experience for us and took care of every one personally.

My sincere thanks to Dr. Christiaan van der Tol and Dr. Mhd. Suhyb Salama who supervised and guided me throughout my research. Your motivation, timely follow-ups and critical comments have made this thesis to take a shape. You navigated me in right direction whenever you thought I needed it. I really learned lot from you and I appreciate your every help.

I received great help from Caroline Lievens, Watse Siderius, Peiqi Yang and Jan Hofste. Your support and assistant in spectroscopic measurements and data collection was highly valued.

I thanks to department of Natural Resources of the Faculty of Geo-information Science and Earth observation, University of Twente for availing a facility to grow plants for my study.

I would also like to express my large thanks to Forschungszentrum Jülich for providing PAM instrument without which validation of my results was not possible.

I thank to Raghav, Kaaviya and all ITC friends who made my time at ITC easier and memorable. Without you all, the stay at this whole new world would have been very tough.

At last, I thank to my parents and family for endless sacrifices, unconditional love and prayers. Although you hardly understand what I research on and what writing thesis is, you support and motivate me every moment. All my achievements are due to you, I am very lucky to have you all with me.

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ASD	ASD FieldSpec Pro FR spectrometer
ChlF	Chlorophyll fluorescence
EVI	Enhanced Vegetation Index
FLD	The Fraunhofer Line Depth
FWHM	Full width at half maxima
NIR	Near infra-red
NDVI	Normalize Difference Vegetation Index
NPQ	Non-photochemical quenching
PQ	Photochemical quenching
PS I	Photosystem I
PS II	Photosystem II
PAM	Pulse Amplitude Modulation Fluorometer
PAR	Photosynthetically active radiation
Q_A	Plastoquinone
R_a	Apparent reflectance
SIF	Sun induced chlorophyll fluorescence
V_{cmax}	Maximum carboxylation capacity of Rubisco

1. INTRODUCTION

1.1. Background

1.1.1. Vegetation Stress

The world's human population is growing rapidly and is leading to unprecedented demand for food and natural resources. To meet these demands, it is necessary to increase agriculture production. At present, agriculture production is under the threat of environmental stress, where stress is "any external factor that affects or blocks the plant's metabolism, growth or development" (Lichtenthaler, 1995). Environmental stress factors include: temperature, light, wind, availability of water and nutrients etc. As photosynthesis is the underlying process for plant growth and an important indicator of the plant efficiency, it is the most affected process by environmental stress factors. To improve the plant growth, efficiency and essentially the agriculture production, it is necessary to study the effects of environmental stress on photosynthesis and pre-symptomatic detection of stress. Therefore, over last two decades, researchers have been working on the timely detection of stress responses of vegetation at the regional and global scale.

Stress to the plant could be apparent in morphological or physiological properties of the plant and can be detected by studying these properties. At present, various methods exist for identification of vegetation stress. Methods such as measuring the rate of photosynthesis, respiration, transpiration, ratios of the photosynthetic pigment or concentration of stress metabolites are the classical eco-physiological methods (Lichtenthaler, 1995). These methods have helped to understand the mechanism of photosynthesis, but these are only applicable at leaf scale, they are not suitable for canopy, field or regional scale. Other methods such as optical remote sensing based spectral indices (e.g. Enhanced Vegetation Index (EVI), Normalize Difference Vegetation Index (NDVI), Simple ratio and modified simple ratio etc.) have been used to identify prolonged vegetation stress at regional and global scale. These spectral indices are useful in understanding the seasonal variation or change detection in canopy which occurs on longer time scale. But, as the optical indices are co-related to leaf pigments and measure only greenness of the vegetation not the actual photosynthesis, they have very less or no sensitivity to the short term physiological changes in vegetation (Garbulsky, Peñuelas, Gamon, Inoue, & Filella, 2011).

Recently, an alternative to above mentioned methods have been provided by plant stress physiologist i.e. to use measurements of chlorophyll fluorescence (ChlF)(Meroni et al., 2009). ChlF from plant can provide a direct measure of photosynthesis and could be related to the short term variations in plant physiology (Maxwell & Johnson, 2000). ChlF can be used at leaf scale as well as regional scale and could be very useful in pre-symptomatic vegetation stress detection which is essential for improving agriculture production.

1.1.2. Leaf Chlorophyll Fluorescence

The ChlF in photosynthetically active plant arises from green tissues in response to the photosynthetically active radiation (PAR) i.e. visible light ranging from 400 nm to 700 nm wavelength. When a chlorophyll pigment molecule absorbs a photon, it undergoes a transition from S_0 to the first electronic excited singlet state S_1 or the higher energy states depending upon excitation energy. At higher energy state these chlorophyll pigment molecules are extremely unstable and therefore they fall back to lower energy level, losing some of the energy very rapidly as heat during internal conversion and some of the energy by re-emitting as a photon of longer wavelength as ChlF (around 680 nm and 740 nm) while returning to the ground state. The wavelength of the emitted fluorescence during this process is always longer than the wavelength of absorbed photons, because a part of excitation energy is lost as heat before the fluorescence photons are emitted.

During the photosynthesis reaction, the ChlF is emitted in competition with the photochemistry and heat dissipation. In the chloroplast, probabilities of photochemistry, fluorescence and heat dissipation sum up to one (van der Tol, Verhoef, & Rosema, 2009). Thus, if one of them increases, the sum of other two must decrease. An increase in fluorescence means a decrease of the sum of the probabilities for photochemistry and heat dissipation (Rosema, Snel, Zahn, Buurmeijer, & Van Hove, 1998). Therefore, the interdependence of these three processes forms the basis for ChlF to be used as a probe to photosynthesis and indicator of stress induced change in plant (Maxwell & Johnson, 2000). It can be seen from Figure 1 that out of 48-94 % of PAR absorbed by plant leaf, 75-97% is dissipated as heat, 3-5% is emitted as fluorescence and 0-20 % of PAR is used for photochemistry.

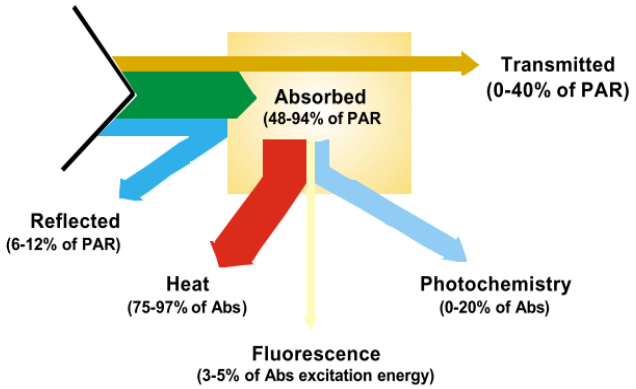


Figure 1 Schematic view of energy partitioning of incident radiation at plant leaf.
Source: Pablo J. Zarco-Tejada (2000)

In oxygenic photosynthetic green plants, chlorophyll-a is the main light harvesting pigment. Chlorophyll-b and carotenes act as accessory pigments. These pigments are part of light harvesting protein complexes known as photosystems. There are two photosystems which work in conjugation i.e. photosystem II (PS II) and photosystem I (PS I). ChlF at room temperature comes from these two protein complex photosystems. Photosystem II is responsible for emission of ChlF at 685 nm while photosystem I for ChlF at 740 nm. As the emission of florescence is a biophysical process, the characteristics of fluorescence emission is determined by the factors such as the wavelength of ChlF excitation, available PAR, concentration of light harvesting pigment molecules, electronic state of pigments, light use efficiency, electron transport rate, enzymes involved in carbon metabolism, effect of the environmental stress etc. (Krause & Weis, 1991; Maxwell & Johnson, 2000; van der Tol et al., 2014). Variation in any one of these factors can result in increase or decrease in ChlF emission depending upon physiological status of plant. For example, under low light condition, an increase in light intensity leads to an increase in steady state ChlF and a decrease in photochemistry. Under high light conditions, however, ChlF and photochemistry both decrease with increased light intensity and moisture stress in response to protective mechanisms such as deactivation of antenna, activation of xanthophyll cycle and non-photochemical protection (van der Tol, Verhoef, & Rosema, 2009).

1.1.3. ChlF Measurement

Depending upon excitation light source, ChlF is classified into active and passive fluorescence. In active ChlF, laser beam, halogen light or LED light of PAR capable of stimulating the photosynthesis are used as source for ChlF excitation. In most cases, blue light and red light of wavelength 450 nm and 650 nm respectively are used because of greater absorption of these lights by photosynthetic pigments. In passive ChlF, fluorescence is excited by solar radiations absorbed by the plant. This ChlF is called as sun induced chlorophyll fluorescence (SIF).

The ChlF emitted by the plant is a very weak signal and it is added to the reflected solar radiation. Therefore, the observed apparent reflectance (R_a) of the plant has a contribution from both, reflected radiation and emitted fluorescence (Meroni et al., 2009). To use this signal of ChlF embedded in R_a as a proxy to the photosynthesis, it is necessary to decouple it from the reflectance and to quantify it. Current methods used for quantification of fluorescence signal are based on radiance approach and reflectance approach (Meroni et al., 2009). The radiance approach is founded on decoupling the fluorescence from reflectance at certain wavelengths (The Fraunhofer absorption lines) where solar spectrum is attenuated. In visible and near infra-red (NIR) region, the solar spectrum has three main Fraunhofer absorption lines: one due to hydrogen absorption at 656.4 nm and other two caused by oxygen absorption i.e. O_2B and O_2A at 687.0 nm and 760.4 nm respectively. The Fraunhofer Line Depth (FLD) method, which is the principle method based on radiance approach has been widely used for quantification of sun induced chlorophyll fluorescence (SIF) at leaf level and regional scale. Performing the FLD method in field requires instruments with very high spectral resolution and full width at half maxima (FWHM) i.e. less than 1 nm (~0.1 nm) to retrieve fluorescence in the narrow Fraunhofer absorption line of the solar spectrum. The reflectance approach is primarily based on quantification of ChlF peak in the R_a. They are quite simple and are often used to quantify artificial light induced ChlF (Meroni et al., 2009). Campbell, Middleton, Corp, & Kim (2008) proposed that the relative amount of ChlF in R_a depends on plants physiological status and that by studying R_a of the plant, it is possible to draw conclusion about plants health.

Apart from these, another ChlF measurement technique exists called Pulse Amplitude Modulation (PAM) fluorometery. PAM is the most used active fluorescence technique, in which ChlF is excited using selective modulated laser beam and relative variation in the steady state chlorophyll fluorescence yield in plant is measured. The measurements using PAM are good indicators of plant photosynthesis efficiency but are limited in their use at leaf scale only.

1.2. Summary and Problem definition

At present, spectral measurements of ChlF are performed by using hyper-spectral spectroradiometers. For accurate quantification of ChlF using hyper-spectral instruments, one need measurements of high spectral resolution i.e. of wavelength <1 nm and good retrieval algorithm. Also, these spectral measurements are often need to be coupled with integrating sphere or assembly consist of short/long-pass filters at illumination and detector end respectively. These technical requirements makes ChlF measurements expensive, difficult and limits the use of low spectral resolution instrument in ChlF studies.

It may be possible to measure the change in ChlF using measurements of low spectral resolution spectrometer with high signal to noise ratio, by tracking the ChlF emission in reflected radiance spectrum of plant, without using integrating sphere and short/long-pass filter assembly. The relative change in ChlF at different physiological stages observed in this way could be interpreted as indicator of plant stress.

There are two possible approaches by which ChlF could be detected in reflected radiance.

First, by illuminating the plant leaf with full spectrum of PAR (halogen light) and measuring the reflected radiance difference or apparent reflectance difference between two states where ChlF yield varies significantly.

For example:

On illuminating leaf with intensity E_1, apparent reflectance (R_{a1}) recorded by spectrometer will be composed of reflected radiance (L_{r1}) and fluorescence (F_1), and is given by equation as follows:

$$Ra_1 = \frac{\pi L_1}{E_1} = \frac{\overbrace{\pi^{-1} E_1 \times \rho}^{L_1} + F_1}{E_1} = \frac{\pi Lr_1 + F_1}{E_1}$$

…….Equation 1

where
L_1 is upwelling radiance at illumination intensity E_1 and ρ is reflectance factor.
On changing the illumination intensity from E_1 to E_2, corresponding apparent reflectance (R_{a2}) will be composed of reflected radiance (L_{r2}) and the fluorescence (F_2) induced due to E_2, and is given by equation as follows:

$$Ra_2 = \frac{\pi L_2}{E_2} = \frac{\overbrace{\pi^{-1} E_2 \times \rho}^{L_2} + F_2}{E_2} = \frac{\pi Lr_2 + F_2}{E_2}$$

…….Equation 2

where
L_2 is upwelling radiance at illumination intensity E_2
If we know E_1 and E_2 from white surface (i.e. spectralon panel) measurement, and assuming that reflectance factor- ρ does not changes over the time, change in fluorescence due to change in illumination intensity can be found as follow:

$$Ra_1 - Ra_2 = \frac{\overbrace{\pi^{-1} E_1 \times \rho}^{L_1} + F_1}{E_1} - \frac{\overbrace{\pi^{-1} E_2 \times \rho}^{L_2} + F_2}{E_2} = \frac{F_1}{E_1} - \frac{F_2}{E_2}$$

…….Equation 3

This change in R_a can potentially be attributed to a change in fluorescence with two intensities and can be interpreted as an indicator of vegetation stress, but a key assumption is that the reflectance factor is constant.
In second method, the ChlF could be detected by exciting the ChlF with a light whose spectrum does not overlap with the ChlF emission and measuring the reflected radiance at ChlF emission wavelengths. Such illumination sources are blue and red light which are absorbed by the plant most efficiently and they do not overlap with ChlF emission bands.
Therefore, in the present study, attempt has been made to detect the change in steady state ChlF fluorescence by using measurements of low spectral resolution spectrometer (without using integrating sphere and short/long-pass filter assembly) and to investigate the change in ChlF as an indicator of the plant stress. Halogen light and LED light (LED; light with blue and red LED designed to simulate plant growth by emitting selective electromagnetic radiations which are best for photosynthesis) were used as source of ChlF excitation. To induce the change in steady state ChlF, two conditions were selected where ChlF varies significantly, first was variation of illumination intensity and second was dark to light transition of plant. To evaluate the possibility of this method to be used for stress detection, we studied effects of high temperature stress on change in ChlF during dark to light transition of plant
The study was performed on two species of plant, C_3 species- Spinach (plant that uses C_3 carbon fixation pathway) and C_4 species- Corn (plant that uses C_4 carbon fixation pathway). C_3 and C_4 plants shows different physiological reposes to environmental stresses due to their physiological and biochemical mechanisms to acclimatize the stress (Yamori, Hikosaka, & Way, 2014). Therefore, through this study the response of C_3 and C_4 plant to the high temperature stress was also investigated.

1.3. Research Identification

1.3.1. Research objectives

The main objective of this research is to detect the change in steady state ChlF using measurements of low spectral resolution spectrometer and to investigate the effect of high temperature stress on transient change in ChlF on illumination of dark adapted C_3 and C_4 plant.

The specific objective of research are

1. To detect the spectral change in steady state ChlF on illumination of leaf at two light intensities.
2. To detect the transient change in ChlF on illumination of dark adapted plant.
3. To investigate the effect of high temperature stress on the transient change in ChlF on illumination of dark adapted C_3 and C_4 plant.
4. To develop a measurement protocol for detecting changes in ChlF at leaf level in the laboratory setting.

1.3.2. Research questions

Based on the above research objectives, following research questions has been formulated

1. Is it possible to detect the spectral change in ChlF from radiometric measurements of low spectral resolution spectrometer?
2. How does the illumination intensity affect ChlF?
3. How does the ChlF spectrum change during dark to light transition?
4. What are the effects of high temperature stress on the ChlF transient?

2. LITERATURE REVIEW

2.1. Application of ChlF in stress physiology

ChlF is a pre-symptomatic, non-destructive and rapid technique for detection of plant physiological status. Several studies have demonstrated the relationship of ChlF with photosynthesis (Rosema et al., 1998; van der Tol et al., 2008; Zarco-Tejada et al., 2013) and also the use of ChlF in improving the crop production (Baker & Rosenqvist, 2004).

Rosema et al. (1998) studied the relationship between ChlF and photosynthesis using combined measurements of laser induced ChlF and CO_2 exchange. The study reported that under high light conditions and temperature stress, ChlF decreases and the ChlF yield is strongly affected by electronic state of photosystems and stress condition. In year 2000, Flexas, Briantais, Cerovic, Medrano, & Moya - measured the diurnal change in ChlF along with the photosynthetic performance using gas exchange in well-watered and water-deficit plant and reported that, during diurnal cycle, steady state ChlF has an inverse relationship with light intensity due to increase in non-photochemical quenching in water deficit plant. Zarco-Tejada et al. (2013) reported that water stress produced during drought in vineyard plants can be detected using SIF. They found that vines under the same growth stage but with different water treatments exhibit a positive relationship between ChlF and photosynthesis

The effect of different illumination intensities on ChlF yield in a lettuce plant were observed by Fu, Li, & Wu (2012). Effects were observed in terms of non-photochemical quenching (NPQ- decrees in ChlF due to increase in heat dissipation), photochemical quenching (PQ-decrease in ChlF due to increase in electron transport at the PS II reaction centre) and quantum yield of PS II photochemistry. Reported results show that NPQ increases with illumination intensity, and drops at high illumination intensity. A decrease in quantum yield of PS II photochemistry with an increase in illumination intensities was also observed.

Brestic & Zivcak (2013) studied the effect of high temperature on ChlF and observed that ChlF fluorescence decreases due to inhibition of photosynthesis on exposing plant from 35˚C to 40˚C of temperature stress. The study concluded that inhibition of photosynthesis is due to damage of PS II caused by temperature rise and due to inhibition of RuBP resulting in decreased CO_2 assimilation from elevated temperature.

High spectral resolution reflectance and fluorescence measurement associated with different nitrogen, carbon dioxide and ozone treatments were studied by Campbell, Middleton, McMurtrey, Corp, & Chappelle (2007). They used reflectance and fluorescence indices to identify the stressed and unstressed vegetation condition. Study concludes that the fluorescence ratio (530 nm/740 nm) is a more prominent indicator of vegetation stress than reflectance ratio. ChlF has successfully detected the effect of heavy metal concentration on photosynthesis (Kancheva, Borisova, & Iliev, 2008) and also the water stress response between conventional and transgenic soya been plant (Caires et al., 2010).

Baker & Rosenqvist (2004) reviewed the use of ChlF to investigate the effect of environmental stressors on crop production and to identify crop varieties that are tolerant to stressors. In their study crops under drought, high temperature, freezing and nutrient stress were studied using active ChlF measurement techniques.

Various modelling efforts have been undertaken to understand the relation between ChlF and photosynthesis. van der Tol, Verhoef, & Rosema (2009) presented a leaf biochemical model for steady state ChlF and photosynthesis at leaf level for C_3 and C_4 vegetation's. They explained the behaviour of photochemistry and fluorescence in response to irradiance and carbon dioxide concentration. The model results show that photochemistry drops and ChlF initially increases on increasing irradiance due to photochemical quenching until carboxylation becomes enzyme limited, fluorescence and photochemistry decreases with decrease in carbon dioxide concentration, at high irradiance fluorescence decreases in stress condition and to calculate the actual photosynthesis maximum carboxylation capacity (V_{cmax}) is an

important parameter. van der Tol, Verhoef, Timmermans, Verhoef, & Su, (2009) explained the role of V_{cmax}, chlorophyll content, vegetation structure, leaf area index as an important vegetation parameter in deriving canopy leaving SIF in the SCOPE model. These models are of great importance in studying the relation between light use efficiency and canopy leaving fluorescence under different vegetation stress conditions.

2.2. ChlF signal in remotely sensed data

The ChlF signal emitted by plant is embedded in its reflected radiations. Buschmann & Lichtenthaler (1999) measured the reflectance at ChlF emission wavelengths in vivo leaves with different chlorophyll concentrations. They found that reflectance at ChlF emission wavelengths has inverse relationship with chlorophyll concentration.

Zarco-Tejada et al. (2000a) measured the effect of ChlF on Ra using halogen lamp as illumination source and long pass filter (wavelength >695 nm) to separate reflectance and fluorescence. They studied reflectance spectra with fluorescence and without fluorescence and found that Ra is affected by ChlF. They used reflectance difference method (Figure 2) to separate the ChlF signature from Ra and plotted the time dependent change in Ra at 690 nm and 740 nm which followed the Kautsky curve measured by PAM-2000 fluorometer.

Zarco-Tejada et al. (2003) demonstrated that the double peak feature 690 nm and 720 nm in Ra is the effect of ChlF rise due to low chlorophyll content in stressed vegetation and this feature can be used to detect vegetation stress. Dobrowski et al.(2005) proposed two ratio indices R690/R600 and R740/R800 to quantify the effect of stress on plant.

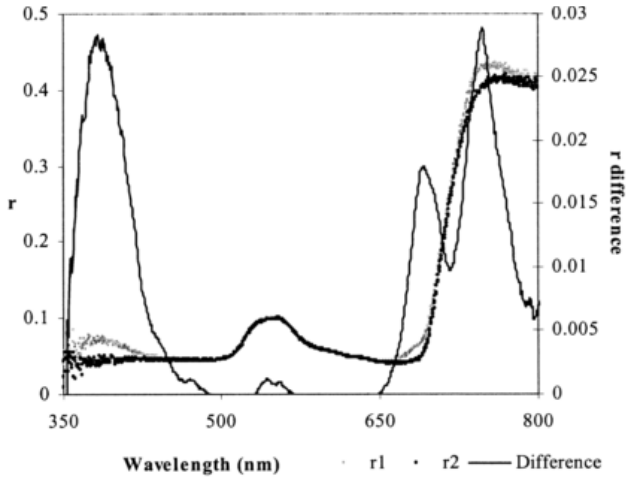


Figure 2 Reflectance difference spectrum, which demonstrate that ChlF emission band affects the reflectance. Source: Zarco-Tejada et al. (2000)

The relative contribution of reflectance and ChlF to the apparent reflectance in red and near infra-red was quantified by Campbell et al. (2008). Measurements were performed using halogen lamp as light source and long pass filters to separate fluorescence from reflectance. They found that, in all measurements, R_a was higher than reflectance and estimated that steady state ChlF at 685 nm contributes 10-25 % to R_a while contribution from 740 nm is about 2-6 %. The study also revealed that the relative contribution of ChlF varies with plant species and stress induced changes.

2.3. ChlF transient

On sudden exposer to light, a dark adapted leaf shows fast fluorescence increase from minimum level *O* (also called as F_0) to maximum level *P* (i.e. F_m) within 500 ms and then it slowly decreases to steady state

terminal fluorescence level *T* (i.e. *Ft*) within 3-5 minutes or depending upon plant's physiological status (Pandey & Gopal, 2012). These time dependent (transient) changes in yield of ChlF are known as 'Kautsky phenomenon' or ChlF induction (Cao & Govindjee, 1990).

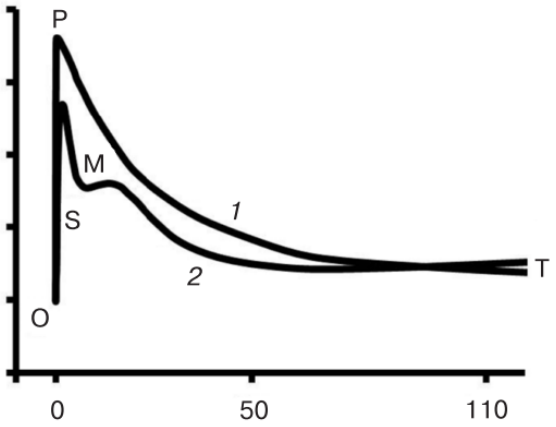


Figure 3 Kautsky curve (Fluorescence intensity in relative unit verses time in ms);
Source: Stirbet, Riznichenko, Rubin, & Govindjee (2014)

This increase in ChlF during a transition from dark to light has been explained as a consequence of reduction in electron acceptors (Plastoquinone) in electron transport pathway. On absorption of light by PS II reaction centres, excited electrons are passed on to Plastoquinone (Q_A), but once Q_A has accepted the electron, it is not able to accept another until it has passed first electron to subsequent electron carrier. During this period of time, reaction centres are said to be closed and this closure leads to a decrease in the efficiency of photochemistry and corresponding increase in ChlF (Maxwell & Johnson, 2000).

Omasa, Shimazaki, Aiga, Larcher, & Onoe (1987) observed the effects of different light intensities and SO_2 treatment on ChlF transient and found that ChlF intensities at intermediate, peak and steady state stages increases on increasing the light intensity from 50 μmole m^{-2} s^{-1} to 200 μmole m^{-2} s^{-1} and the appearance of peak becomes faster with the increase in light intensity. With respect to SO_2 the treatment, peak intensity of ChlF, *P,* was found to be reduced with slight increase in steady state ChlF. Their study reported that ChlF transient is an important indicator of various reactions of photosynthesis and could be used for detection of plant physiological status.

Briantais, Dacosta, Goulas, Ducruet, & Moya (1996) performed a time resolve study of heat stress on ChlF yield and observed that increasing temperature from 23°C to 50°C induces the quenching of maximum fluorescence (F_m) and increases the minimum fluorescence yield in dark adapted plant (F_0). The cause of the increase in F_0 was that on increasing the leaf temperature the yield of photochemistry decreases giving rise to increased yield of F_0. Recently, Stirbet, Riznichenko, Rubin, & Govindjee (2014) reviewed the Chlorophyll fluorescence transient concept explaining all stages of OJIPSMT transient, where O is the minimum ChlF level, J and I are intermediate inflections, P is peak; S is semi steady state level, M is maximum and T is terminal steady state level. The review explains that O to P rise in transient is due to the reduction of Q_A and has a life time of 300 ms -500 ms while P to T decline is the result of various quenching mechanisms such as NPQ/ PQ and P-T stage can last for 3-4 minutes.

3. MATERIALS AND METHODS

To answer the research questions mentioned in Chapter 1, the methodology was divided into following three experiments.

Experiment 1). To detect the relative change in ChlF at two illumination intensities

Experiment 2). To detect the transient change in ChlF on illumination of dark adapted plant.

Experiment 3). To study the effect of increase in temperature on the transient change in ChlF after illumination of dark adapted plant.

Each experiment consisted of two sub experiments- one using a halogen lamp and another using LED light as a light source. A halogen lamp was chosen because of its ability to reproduce the solar illuminations in PAR range and its use in previous studies, while LED light was selected because it contains blue and red lights which that have ChlF excitation wavelengths. With LED light ChlF emission spectra at 740 nm was possible to be detected without any use of a short/long pass filter.
All experiments were performed at leaf level in a controlled environment. During each experiment a leaf of a plant was illuminated with a light source and the reflected radiance spectra were recorded using ASD in conjugation with PAM measurement to validate the results.

3.1. Materials

3.1.1. Plant

Zea mays (Corn) and *spinacia oleracea* (Spinach) plants were chosen for experiment due to their use in similar studies (e.g. Schmuck & Moya (1994); Damm et al. (2010)). Corn is C_4 plant (plant that uses C_4 carbon fixation) and follows a life cycle of 4-5 month. Corn grows in mid cold to warm temperature. Due to Corn's rapid growth and its shallow root system, it was possible to grow it in a controlled environment at laboratory. Spinach is C_3 plant (plant that uses C_3 carbon fixation) and follows a life cycle of 4-5 months similar to Corn. Spinach can tolerate low temperatures and was possible to grown it in moderate light conditions of winter season.

a) b)

Figure 4 *zea mays* (a) and *spinacia oleracea* (b) used in study

Both corn and spinach seeds were sown in small pots during first week of September and grown in controlled environment with average daily temperature of 20°C-25°C and average daily photosynthetic radiation of 300-400 μmole m^{-2} s^{-1} (Intermediate growth light intensity). PAR was provided using LED plant grow light. Plants were kept well watered every 3 days and nutrients were provided using liquid

nutrient mixture every 15 days. Day and night cycle was maintained as of natural cycle of 10 -12 hours of day and 10-12 hours of night using automatic timer.

3.1.2. Spectroradiometer

Leaf spectral measurements were performed using ASD FieldSpec Pro FR spectroradiaometer (Figure 5). With a spectral range of 350 nm to 2500 nm, ASD has a spectral resolution of 3 nm in VNIR and 10 nm in SWIR bands. It possesses sampling interval of 1.4 nm in the VNIR and 2 nm in SWIR range and is interpolated to 1 nm for total of 2151 channels. The ASD has three detectors to complete the spectral range which it offers: the silicon photodiode detector covering 350 nm to 1000 nm range, and two InGaAS detectors one for 1000 nm to 1800 nm and the other for 1800 nm-2500 nm.

Figure 5 ASD FieldSpec Pro FR spectroradiaometer

The fibre optic of ASD had a field of view of 25°. The instrument acquires data in the form of digital number and converts it to reflectance based on white panel reflectance and dark current measurement. The conversion from DN to reflectance is done using ASD Field Spec Pro software. The instrument specification has been taken from Analytical Spectral Devices (2002).

3.1.3. Pulse amplitude modulation fluorometer (PAM)

The steady state ChlF measurements were performed by using Miniature Pulse Amplitude Modulation Fluorometer (MINI PAM-2000) of the Forschungszentrum Jülich, manufactured by Heinz Walz GmbH, Effeltrich, Germany. PAM is the most widely used instrument in basic and advanced fluorescence studies. It uses modulated ChlF excitation light which passes a short pass filter (<670 nm) and emitted ChlF is recorded at photo detector which is protected by long pass filter (>700 nm). Photosynthetic yield is calculated using a single saturating pulse which reduces all reaction centres and suppresses photochemical yield to zero inducing maximum fluorescence yield. PAM consists of a leaf clip to hold the leaf and fibre-optic cable attached to light source to illuminate the leaf and to record the ChlF signal. PAM leaf clip is equipped with PAR sensor which helps in calculating apparent electron transport rate. Using actinic light PAM is able to measure the light response curve and fluorescence induction curve. The PAM specification has been taken from Heinz Walz GmbH, (1999).

3.1.4. Light illumination source

The light illumination source used for all three experiment were halogen lamp of 225 Watts and LED light consisting of blue (λ=460 nm) and red (λ=660 nm) LED. The halogen lamp was capable of producing PAR of 1000 µmole m^{-2} s^{-2} to 1100 µmole m^{-2} s^{-2} when measured at 10 cm distance using a micro quantum sensor mounted on PAM leaf clip. The halogen lamp had an electromagnetic spectrum ranging from 350 nm to 2500 nm. The LED light had intensity of PAR 300 µmole m^{-2} s^{-2} to 400 µmole m^{-2} s^{-2}. Both light sources were tested for their stable light output and LED lights were found to produce more stable output

(Figure 7) than halogen lamp (Figure 6). Also when the spread of light was compared, the halogen lamp light was much collimated whereas LED light was dispersed. On observing LED spectrum it seen that spectrum of LED at 660 nm has small elongation of spectrum in the range of ChlF emission wavelength.

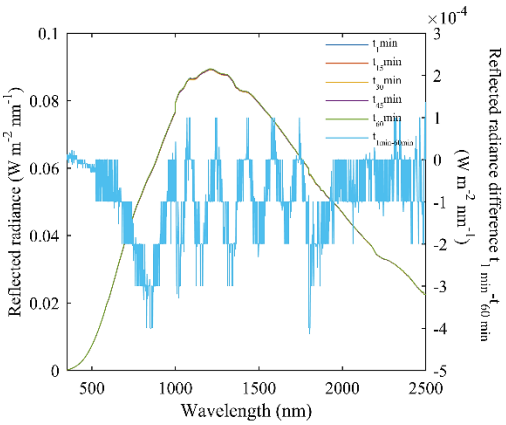


Figure 6 Irradiance from halogen light tested for stable output

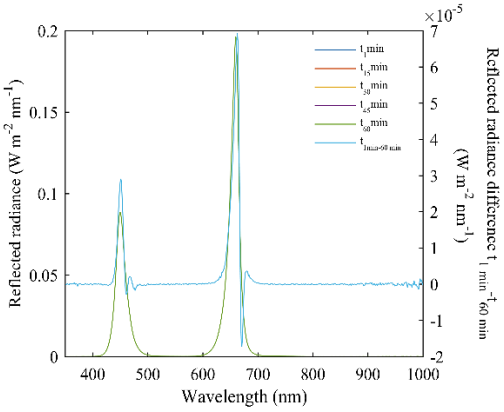


Figure 7 Irradiance from LED light tested for stable output

3.1.5. Dimmer

To control the illumination intensity of the light source, a dimmer was built which was able to vary illumination intensity from lowest to highest or vice versa. The dimmer was marked with scale from 0-10 based on PAR output of halogen light as shown in Table 1. These measurement of PAR were performed at 10 cm distance between light sources using PAR sensor embedded in leaf clip of PAM.

Marking on dimmer	PAR µmole m^{-2} s^{-1}
0	50
1	100
2	150
3	200
4	250
5	300
6	350
7	400
8	450
9	500
10	550

Table 1 PAR output of dimmer at each marking

3.1.6. Hot air blower

In order to see the effect of high temperature on ChlF transient of dark adapted plant, it was necessary to heat the plant. For this purpose hot air blower capable of increasing the temperature of air flow up to 50°C was used. The blower has temperature control to regulate the temperature of airflow.

3.2. Experimental setup

Leaf level experimental setup was designed to collect reflected radiance using ASD and ChlF using PAM at the same time. To eliminate the influence from reflectance other than the leaf under the study, the experiments were performed in a dark room facility of the GeoScience Lab of ITC, University Twente.

This dark room has black painted walls and it is the completely isolated from outside light. During the setup of experiments, care was taken to minimise the influence of light other than illumination source and the platform was covered with black cloth to reduce the background reflectance. The fibre-optic cable of ASD and PAM were mounted together in a holder pointing nadir towards the leaf. The light source connected with dimmer was installed at 45° incident angle pointing at leaf sample. The distance between sample and the lamp was 10 cm while distance between ASD fibre optic and sample was 4 cm providing sampling area of 2 cm diameter.

The plant leaf was positioned facing the fibre optic horizontally. Care was taken to keep leaf exactly under the field of view of the fibre optics cable with minimum inclination. A white reference panel was placed to obtain irradiance measurements of the illumination source with similar geometry as that of the leaf. The ASD was configured to reduce noise by setting number of black current spectra, number of white reference and number of sample spectra averaged to 25.

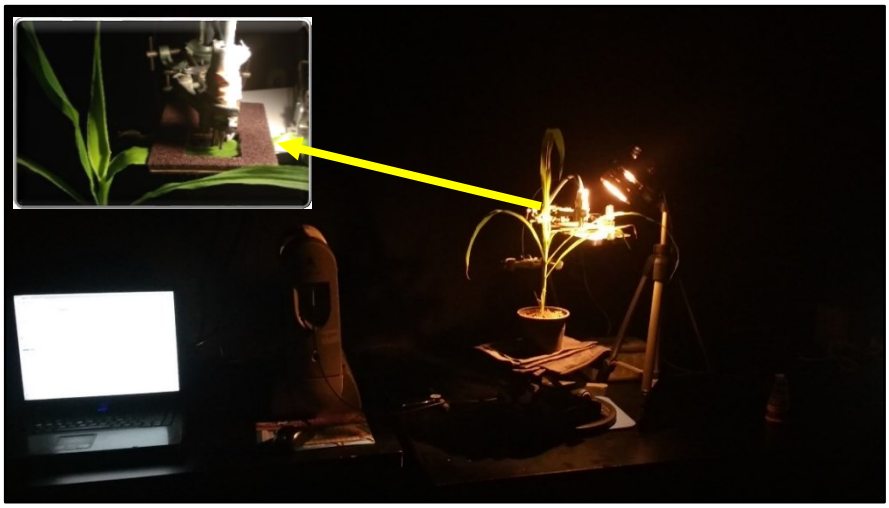


Figure 8 Experimental setup

The design of experimental setup (Figure 8) has been adopted from Amorós-López et al.(2008); Corbin (2015); Atherton, Nichol, & Porcar-castell (2016).

3.3. Methodology

3.3.1. Experiment 1: To detect the relative change in ChlF at two illumination intensities.

To investigate whether it is possible to measure the spectra change in ChlF using low spectral resolution spectrometer, an experiment was performed in which plant leaf was illuminated with two light intensities and the reflected radiance recorded using ASD was observed for the presence of a ChlF signal. The detailed methodology for the experiment is explained in the following steps.

Step 1) Light curve: To investigate the relative change in chlorophyll fluorescence on illumination of plant with two different illumination intensities, it was necessary to define a limit for minimum and maximum illumination intensities at which ChlF yield varies significantly. Such observations can be made using light curve where ChlF yield is plotted against increasing light intensity (Figure 10 and Figure 11). Therefore, the instant light response curves were obtained using light curve programme of the PAM where actinic light intensity was increased from 50 µmole m^{-2} s^{-1} to 500 µmole m^{-2} s^{-1} during 4 minutes in eight steps following each step with 30 second (Rascher, Liebig, & Lüttge, 2000). Prior to light curve, leaves were

dark adapted for 30 minutes to oxidise the electron carriers in photosynthetic tissue which on subsequent exposer to light gives maximum ChlF (P.J. Zarco-Tejada et al., 2000).
Step 2) Optimization of instrument: Optimization is necessary to increase the response of the detector to the light in a certain spectral region. Therefore calibration and optimization of ASD was performed according to the instructions given in instrument manual Analytical Spectral Devices (2002). A spectralon panel was used for optimizing and taking white reference measurements.

Step 3) Sample positioning and spectral measurement: In vivo plant leaf was positioned under the ASD fiber optic at the distance of 4 cm and the adaxial surface of leaf was kept facing horizontally towards fiber optics with minimum inclination. Care was taken to limit self-shading of the system. For first spectral measurements, the sample was illuminated with low illumination intensity (i.e. 50 μmole m^{-2} s^{-1}) and the reflected radiance spectra were recorded using ASD and the ChlF yield using PAM. White reference measurements were taken before and after each spectral measurement of leaf. The leaf temperature was also recorded using the temperature sensor embedded in the PAM leaf clip. For the second measurement, the sample was illuminated with higher illumination intensity (i.e. 200 μmole m^{-2} s^{-1}) and reflected radiance along with ChlF yield were recorded followed by white reflectance measurement and leaf temperature measurement.

Step 4) Spectral processing: When halogen lamp was used as illumination source, each recorded reflected radiance spectrum was converted into reflectance by normalising it with incident irradiance measured at respective light intensity using spectralon panel. The reflectance spectrum was then smoothed using a Savitzky–Golay second order polynomial least-square function to reduce the spectral noise (Zarco-Tejada et al., 2000a). Further, to investigate the presence of ChlF on Ra, reflectance difference spectra were obtained assuming the ChlF yield increased during the illumination from low intensity to high intensity.
The whole experiment (steps 1-4 described above) was repeated using LED light as an illumination source, except that in step 4, reflected radiance spectrum was smoothed and normalised with reflected radiance of 660 nm. The spectrum then observed for presence of a ChlF peak at 740 nm.

3.3.2. Experiment 2: To measure the transient change in ChlF after illumination of dark adapted plant.

The transient ChlF on sudden illumination of dark adapted plant can provide important information regarding various biochemical reactions involved in photosynthesis. This transient of ChlF has been studied by using various high temporal and spectral resolution instruments to track the fast variations in initial stages of photosynthesis on illumination. It may be possible to study these transient changes in ChlF by using low temporal and spectral resolution spectrometer. This could provide information on slow ChlF induction which lasts for few seconds to few minutes. Therefore the experiment to track transient changes in chlorophyll fluorescence of a leaf was performed as follows.

Step 1) Induction curve: To understand the time required to attain steady state ChlF, it was necessary to plot the time response of ChlF upon illumination of dark adapted plant leaf. Therefore, ChlF induction curve was plotted using induction curve program of PAM. The methodology for induction curve has been adopted from Heinz Walz GmbH (1999) and Pandey & Gopal (2012).

Step 2) Optimization of instrument: Calibration and optimization of ASD was performed according to the instructions given in the instrument manual Analytical Spectral Devices (2002) and similar to the process done as in experiment 1 step 2.

Step 3) Sample positioning and spectral measurement: The leaf was positioned under the fibre optic at the distance of 4 cm and the adaxial surface of leaf under study faced fibre optic horizontally with minimum

inclination. The sampled leaf was dark adapted for 30 minutes. After 30 minutes, the leaf was exposed to light with 200 μmole m^{-2} s^{-1} and transient reflected radiance and ChlF were measured in conjugation with each other using ASD and PAM respectively. The reflected radiance and ChlF were measured for the time needed to achieve steady state which was obtained from the induction curve. The temporal resolution of the radiance and ChlF measurements was two seconds. After completion of the induction time, white reference measurements were recorded for each set of experiment. Leaf temperature was also recorded at start and end of the spectral measurement using temperature sensor in PAM leaf clip to verify that the temperature was constant during the experiment.

Step 4) Spectral processing: Each recorded apparent reflectance spectrum was smoothed using a Savitzky–Golay second order polynomial least-square function to reduce the spectral noise.
The whole experiment (steps 1-4 described above) was repeated using LED light as an illumination source.

3.3.3. Experiment 3: To measure the effect of increase in temperature on the transient change in ChlF after illumination of dark adapted plant.

Stress induced changes can damage the photosynthetic pigment and the mechanism of photosynthesis. These changes can be seen in different stages of ChlF transient. Therefore, to see whether the effect of high temperature stress could be tracked in ChlF transient by using measurements of low temporal and spectral resolution spectrometer, the methodology was adopted as follows.

Step 1) Dark adaptation and heating of the plant: In order to see the effect of high temperature on transient change, a plant was dark adapted for 30 minutes and at the same time entire plant was exposed to high temperature air flow using hot air blower. The plant was heated up from room temperature to 40°C (+/-5°C) for 30 minutes. The temperature of airflow was controlled using regulator of the blower and the temperature of leaf was measured using temperature sensor embedded into PAM leaf clip. The leaf temperature was also monitored continuously by portable infrared thermometer.

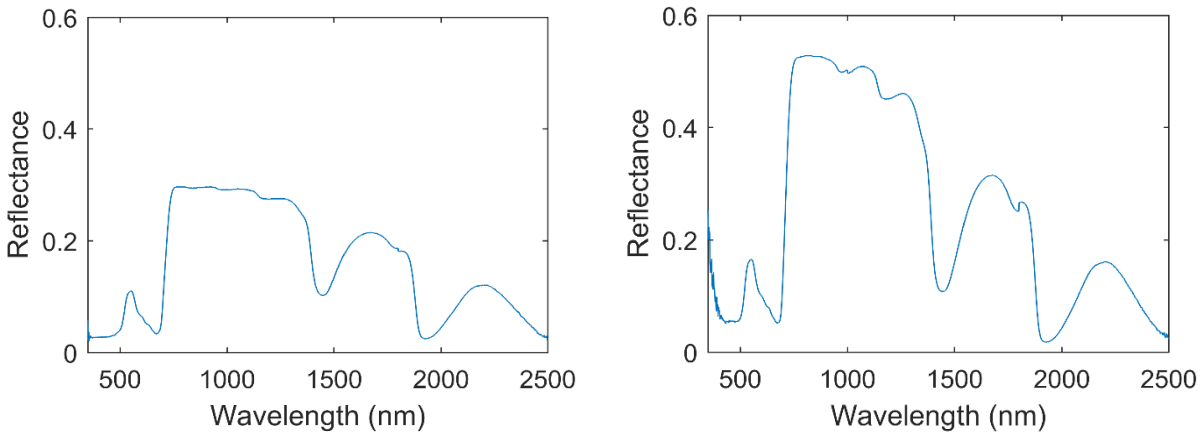
Step 2 to 4 were repeated in the same manner as in experiment 2. The whole experiment was performed using both the halogen light as well as LED light as illumination source.

4. RESULTS AND DISCUSSION

This chapter presents the results of spectral measurements, data processing and analysis for all three experiments. The results of the first experiment illustrate the possibility to detect the spectral change in ChlF using measurements of low spectral resolution spectrometer. In this experiment, the reflected radiance spectrum on illumination of a plant leaf with two light intensities were analysed for presence of a ChlF signature. Results of the second experiment show the transient change in reflected radiance and ChlF on sudden exposer of pre-darkened plant. Lastly, the effect of heat stress on transient change in ChlF detected using low spectral resolution spectrometer has been discussed in third experiment's results. The results explained here belongs to the same sample for all three experiments.

4.1. Reflectace from Corn and Spinach

In vivo reflectance spectra from green leaf of Corn and Spinach obtained using ASD over spectral range of 350 nm to 2500 nm has been shown in Figure 9. Both the reflectance spectrums were recorded at same irradiance of 200 µmole m^{-2} s^{-1}.



a) b)

Figure 9 Typical reflectance spectrum of a) Corn and b) Spinach

The reflectance spectra of Corn and Spinach are somewhat similar in shape but each of these species also displays different spectral properties. These differences are visible in NIR portion of spectrum. It can be seen from Figure 9 that the reflectance from the spinach leaf is higher than corn, mainly in the near infra-red wavelengths. This could be related to the morphological properties of spinach leaves, as at the time of measurement, spinach leaves were well grown, green and thicker than corn resulting into increased size and length of mesophyll cells along with the enlarged aerial interfaces in the spongy parenchyma which may increase the NIR reflectance (Buschmann & Lichtenthaler, 1999; Rapaport, Hochberg, Rachmilevitch, & Karnieli, 2014)

4.2. Experiment 1: To detect the relative change in ChlF at two illumination intensities.

In order to detect spectral change in ChlF, the reflected radiance spectra were recorded using ASD on illumination of leaf with two different illumination intensities: first using low irradiance and secondly using high irradiance. The illumination intensities were so selected such that a significant change in reflected radiance at chlorophyll emission wavelength can be observed. For this, ChlF light curves were plotted as shown in Figure 10 and Figure 11. The light intensity where maximum ChlF yield was recorded by PAM

was selected as high illumination intensity while the one with which intermediate yield was recorded and which was reproducible by a dimmer was selected as lower illumination intensity.

PAR	F	Fm'	Yield	ETR
2	212	1408	0.849	0.7
25	257	1376	0.813	8.5
75	381	1320	0.711	22.4
172	492	1280	0.616	44.5
232	469	1056	0.556	54.2
298	387	845	0.542	67.8
370	341	701	0.514	79.8
455	298	572	0.479	91.5
580	263	503	0.477	116.2

Table 2 ChlF light curve readings recorded by PAM (For control Corn)

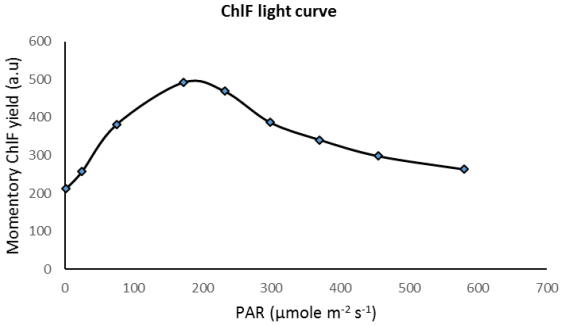


Figure 10 ChlF light curve (for control Corn)

PAR	F	Fm'	Yield	ETR
5	182	1623	0.888	1.9
32	301	1278	0.764	10.3
65	369	1008	0.634	17.3
175	418	903	0.537	39.5
245	496	921	0.461	47.5
293	421	856	0.508	62.5
375	366	761	0.519	81.8
448	311	702	0.557	104.8
572	256	689	0.628	151.0

Table 3 ChlF light curve readings recorded by PAM (For control Spinach)

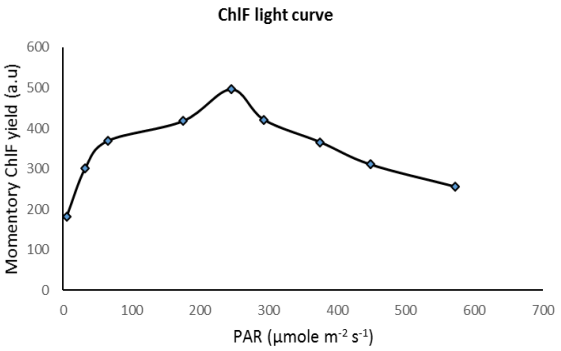


Figure 11 ChlF light curve (for control Spinach)

From Figure 10 and Figure 11 it can be seen that for Corn and Spinach both, the steady state ChlF yield increase on increasing the light intensity till 200-250 µmole m^{-2} s^{-1} and ChlF yield slowly decreases on further increase in light intensity. For current set of results, low intensity of 50 µmole m^{-2} s^{-1} and high intensity of 200 µmole m^{-2} s^{-1} were selected.

4.2.1.1. Halogen light as illumination source

Figure 12 shows the R_a from a Corn and a Spinach leaf obtained using ASD at two light intensities (50 µmole m^{-2} s^{-1} and 200 µmole m^{-2} s^{-1}) when halogen lamp was used as an illumination source. The R_a at low intensity was subtracted form high intensity assuming that at low light conditions, ChlF increases with increasing illumination intensity. The corresponding reflectance difference spectrum was observed for ChlF emission peaks.

As per our assumption, the R_a on illumination with high intensity was expected to rise only at ChlF emission wavelengths and at rest it was supposed to be constant. In this way the corresponding change in ChlF that occurred due to variation in illumination intensity could be tracked in reflectance difference spectrum (P.J. Zarco-Tejada et al., 2000). The reflectance difference spectrum was expected to show a ChlF peaks at 690 nm and 740 nm as seen by Buschmann & Lichtenthaler (1999), Zarco-Tejada et al. (2003) and (Campbell et al., 2008)

But from experiment results (Figure 12), it can been seen that the R_a has increased all over the spectrum, not only at the ChlF emission wavelengths. This increase in R_a is prominent in the entire near infra-red region which cannot be due to the ChlF. On observing reflectance difference spectrum, it can be seen that it does not show any sign of ChlF. These results observed are consistent throughout multiple measurements taken for both corn and spinach.

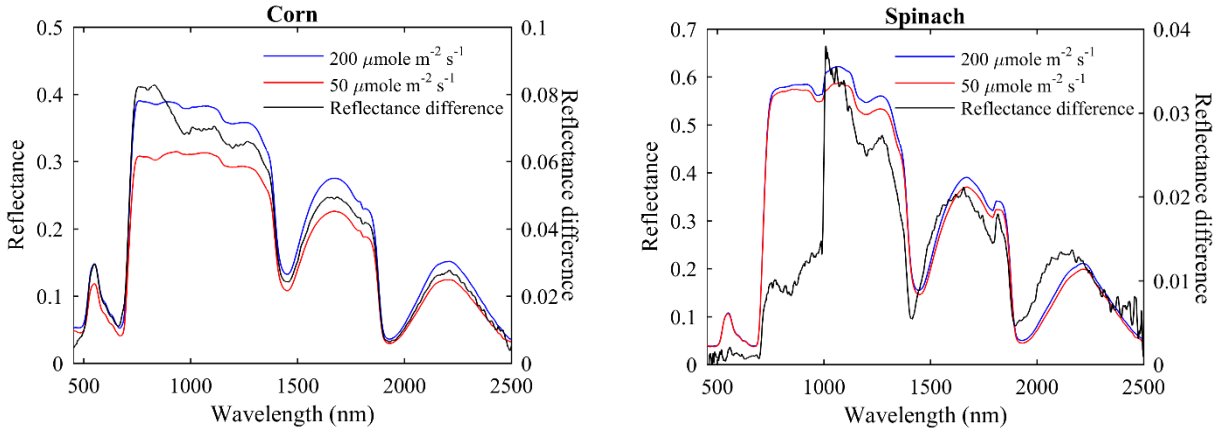


Figure 12 Apparent reflectance at two illumination intensities and corresponding reflectance difference spectrum (For control Corn and Spinach)

The abrupt increase in R_a and the absence of ChlF peaks in the reflectance difference spectrum could be explained through the rise in leaf temperature which was observed during the spectral measurements. When halogen light was used as ChlF excitation source, the transition from low illumination intensity to high illumination intensity rapidly increased the leaf temperature. This rise in temperature was 8°C to 10°C within 3 minute interval which was high enough to heat the leaf and to reduce the leaf water content. As water is the most abundant in healthy leaves, its effect on leaf optical properties is significant. The impact of leaf water content on reflectance could be direct i.e. caused by absorption properties of water or could be indirect, i.e. those linked with leaf properties that changes with hydration or dehydration of leaf (Ollinger, 2011). In most cases due to dehydration, leaf curls inward and shrinks and that may cause a change in leaf geometry. The combined effect of heat and water loss may induce change in morphological properties. It can also affect the opening and closing of stomata and rate of photosynthesis. All these effects may have contributed to the increase in overall reflectance of leaf.

As the increase in R_a at high illumination intensity was significant all over the spectrum, the small ChlF signal which was supposed to be detectable from reflectance difference possibly got masked by combined effect of background reflectance and increased near infrared reflectance.

The measurements from PAM support these results (Table 4). On changing the illumination from low to high intensity, the increase in ChlF yield was recorded at PAM along with the rise in leaf temperature from 22°C to 30°C in 3 min. The rise in steady state ChlF recorded at PAM could be due to reduction of Q_A on illumination with high light intensity or the decrease in photosynthesis due to rise in temperature or both(Dobrowski et al., 2005). The exact reason(s) for this rise could not be confirmed without supplementary information such has photochemical quenching, non-photochemical quenching and gas exchange.

Illumination intensity	Corn		Spinach	
	ChlF (a.u)	Temperature (°C)	ChlF (a.u)	Temperature (°C)
50 μmole m-2 s-1	350	21.7	287	21.5
200 μmole m-2 s-1	570	28.1	561	29.3

Table 4 PAM measurement (experiment 1, light source- halogen light)

4.2.1.2. LED light as illumination source

The Figure 13 shows the reflected radiance from Corn leaf acquired with ASD at two intensities (50 μmole m-2 s-1 and 200 μmole m-2 s-1) when illuminated with LED plant grow light. The recorded spectra were normalised using reflected radiance at 660 nm to make all observations comparable. The presence of ChlF can be seen in these normalized reflected radiance spectra at 740 nm where radiance has peaked

slightly due to contribution from ChlF emission. Also the small change in ChlF emission due to change in illumination intensity is evident in the Figure 13: It shows the increase in ChlF peak on transition from low intensity to high intensity illumination.

These increase in ChlF has also been confirmed by measurements of PAM that showed steady state ChlF rise from 299 a.u to 548 a.u on transition from low to high illumination. When LED light was used as an illumination source, no significant rise in temperature was recorded that could affect the measurements or induce a temperature stress. Therefore the rise in ChlF in this case could be attributed to the progressive saturation of reaction centers in the photosynthesis pathways on illumination with high intensity light.

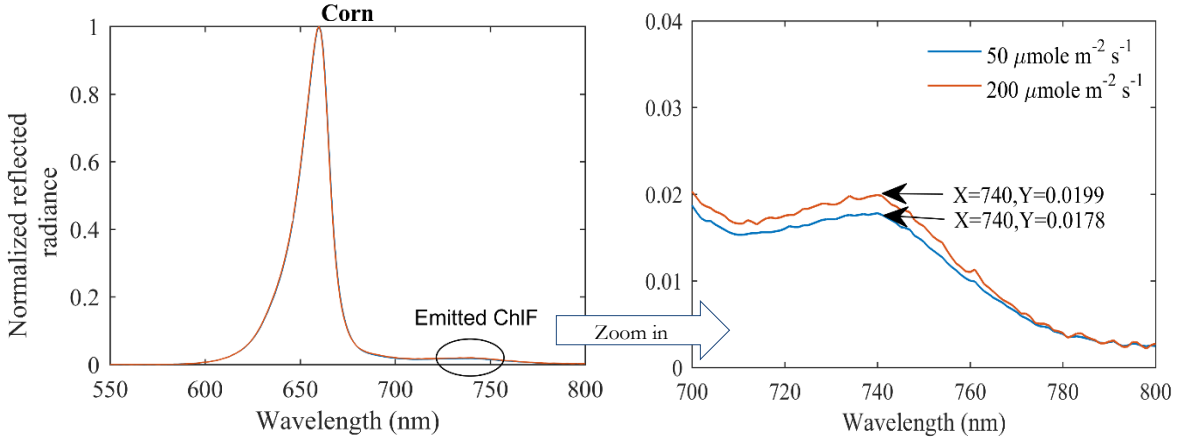


Figure 13 Normalized reflected radiance on illumination with LED light (For control Corn)

The same results as Corn were observed for Spinach (Figure 14). The measurements using ASD were able to track the small change in ChlF at 740 nm. It shows the increase in ChlF emission on illumination with high intensity.

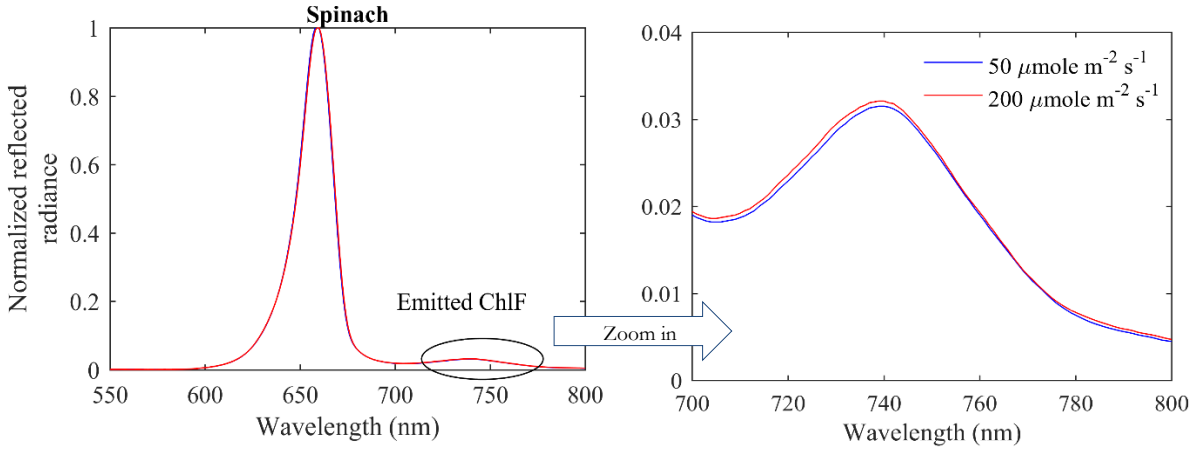


Figure 14 Normalized reflected radiance on illumination with LED light (For control Spinach)

Illumination intensity	Corn		Spinach	
	ChlF (a.u)	Temperature (°C)	ChlF (a.u)	Temperature (°C)
50 µmole m-2 s-1	299	22.1	281	21.3
200 µmole m-2 s-1	548	22.3	509	21.9

Table 5 PAM measurement (experiment 1, light source- LED light)

4.3. Experiment 2: To measure the transient change in ChlF on illumination of dark adapted plant.

The time resolved reflected radiance spectra were recorded using ASD on illumination of pre-darkened leaf with 200 µmole m^{-2} s^{-1} intensity for 3 minutes. The same leaf was illuminated twice, once with halogen light and once with LED light, each time after 30 minutes of dark adaptation. The resulting transient spectra were analyzed for presence of ChlF signal.

4.3.1. Halogen light as illumination source

The result of exposing dark adapted corn leaf to sudden prolonged illumination of halogen light has been shown in Figure 15 from which, transient reflectance for 3 minute and reflectance difference between R_a at time t_1 and t_3 can be observed. The presence of ChlF peaks at 685 nm and 740 nm can been seen in the reflectance difference spectrum which confirms the superimposition of ChlF on reflectance spectrum.

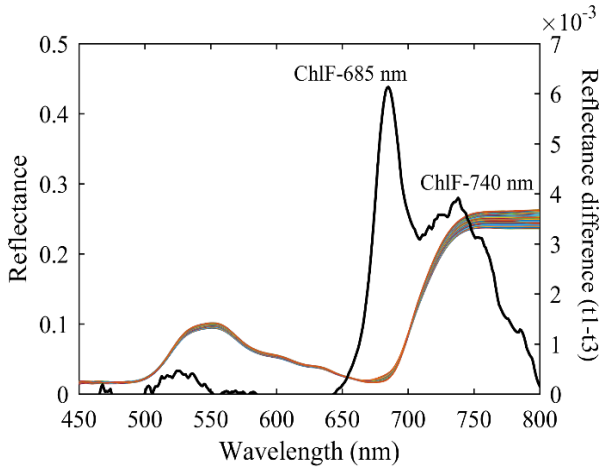


Figure 15 Transient R_a and reflectance difference between Ra at time t_1 and t_3 (For control Corn)

Changes of reflectance bands (R_a 685 and R_a 740nm) affected by ChlF have been shown in Figure 16 where R_a at 685 nm increased at first instance and then gradually decreased till the end of the 3 minutes of illumination. The same trend as at 685 nm was followed by R_a at 740 nm but only for the first 15-20 seconds after which it slowly increased. In both bands, for the first 20 seconds, R_a shows temporal decay similar to the behaviour of the Kautsky curve measured using PAM.

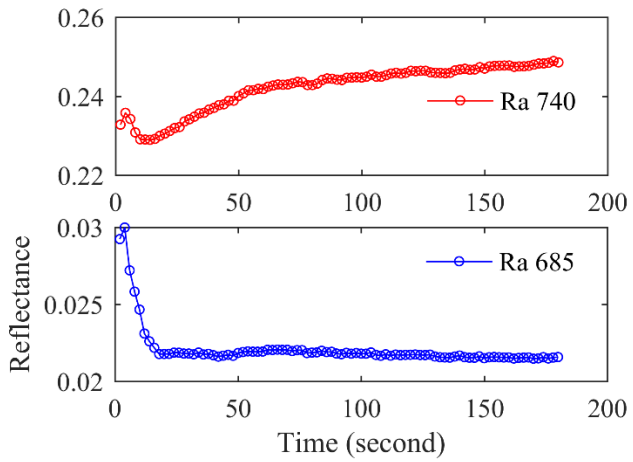


Figure 16 Change in R_a with time at ChlF emission wavelengths (For control Corn).

Figure 17 and Figure 18 show the consecutive reflectance differences of transient R_a. It can be seen that the transient of reflectance difference follows the classical Kautsky kinetics in which ChlF peaks on sudden exposure and then gradually decays due to effects of different quenching mechanisms. The rate of decrease in ChlF is higher for first 16-20 second, after which it slows down and achieves steady state around 50 seconds. Figure 18 shows that these patterns are consistent with the simultaneous measurements of PAM (Figure 19) that recorded sudden increase in steady state ChlF following slow decrease as in Kautsky curve. PAM also recorded the rise in leaf temperature from initial 23°C to 30°C within 3 minutes of illumination.

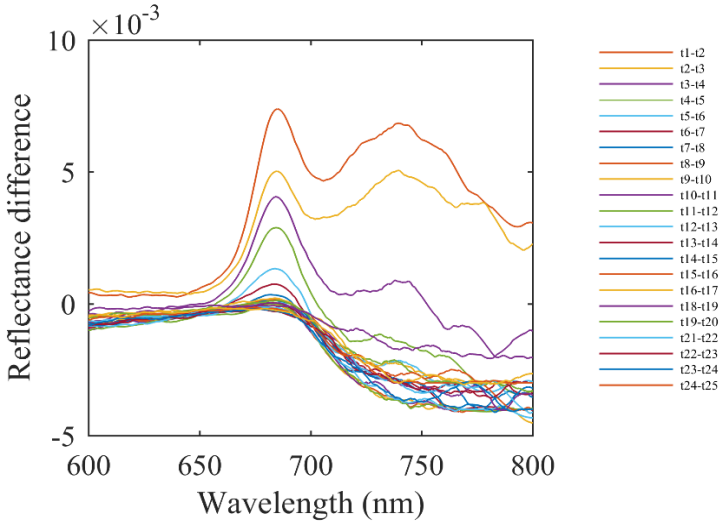


Figure 17 Consecutive reflectance difference in Ra for first 50 seconds
(For control Corn)

On observing PAM measurements (Figure 19), the inflection point labelled PSMT of OJIPSMT induction curve can be seen. PSMT is a slow phase induction curve where ChlF declines to steady state level T in time scale of minutes. The PSMT phase is also visible in transient reflectance difference, but could not be confirmed due to noise in the spectral measurements.

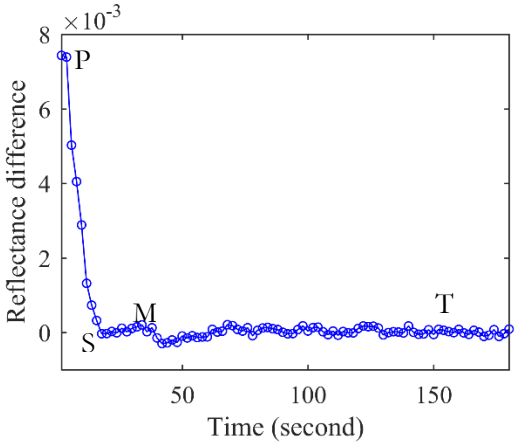


Figure 18 Transient of reflectance difference
(For control Corn)

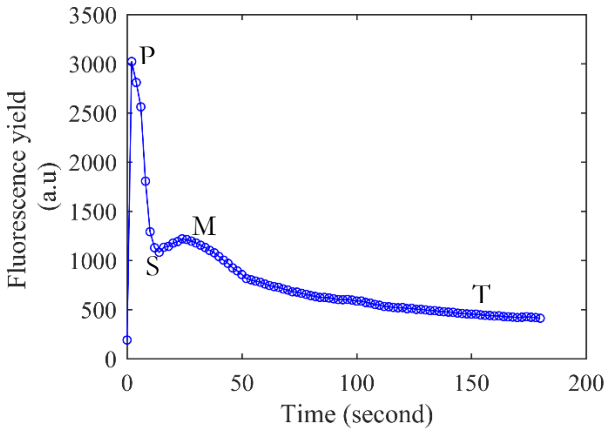


Figure 19 Steady state ChlF measured using PAM
(For control Corn)

Looking at reflectance difference spectrum (Figure 15 and Figure 17), it can be observed that the intensity of ChlF at 685 nm is slightly higher than ChlF at 740 nm. Several researchers such as G. Heinrich Krause & Weis (1984), H. Lichtenthaler, Stober, & Lang (1992) have correlated this variation in peak heights to contribution of ChlF from photosystems and concentration of chlorophyll. According to H. Lichtenthaler

et al.(1992) the leaf with high chlorophyll content shows its ChlF maxima at 740 nm while the one with low chlorophyll content shows its ChlF maxima at 685 nm. The reason is that with high chlorophyll, ChlF at red wavelength (685 nm) is reabsorbed by the leaf pigments reducing the high of ChlF peak. Therefore, the fact that: Corn leaves were not fully grown and green at the time of measurements, it may have resulted into high ChlF peak at 685 nm than 740 nm. This is consistent with Figure 9, which shows a relatively high reflectance in the green for Corn (thus low chlorophyll)

Similar to experiment 1, experiment 2 was also carefully carried out in dark room with precautions to reduce the background reflectance and it was assumed that transient R_a will only change at ChlF emission wavelength and the rest will be constant throughout the 3 minutes of measurements. But in the results (Figure 15), it was observed that: R_a in NIR band increased after 20 seconds till end of the 3 minutes. This rise in NIR reflectance with time could be related to the effect of increasing leaf temperature during measurement. On using halogen lamp as an illumination source, it was found that heat radiated from lamp increases the leaf temperature by 8°C to 10 °C more than the room temperature. The elevated temperature may cause reduction in leaf water and also to change the leaf morphological properties giving rise to R_a in NIR region as the effect was also seen in experiment 1. The effect of rise in NIR reflectance can also be seen on R_a at 740 nm (Figure 16) in which R_a has increased slowly after first 20-25 seconds.

On comparing the results for Spinach with Corn, it can be seen that spinach follows same trend as Corn with small differences. These differences are observed in the ChlF maxima and the time taken to achieve the steady state. The reflectance difference spectra observed for Spinach leaf showed its ChlF maxima at 740 nm and the ChlF peak at 685 nm was found to be lower (Figure 20). This shape of reflectance difference spectra at ChlF emission wavelengths could be due to the presence of higher chlorophyll pigments concentrations as Spinach leaves were fully grown and sufficiently green at the time of measurement, resulting into reabsorption of red ChlF and lowering of ChlF peak at 690 nm relative to the peak at 740 nm.

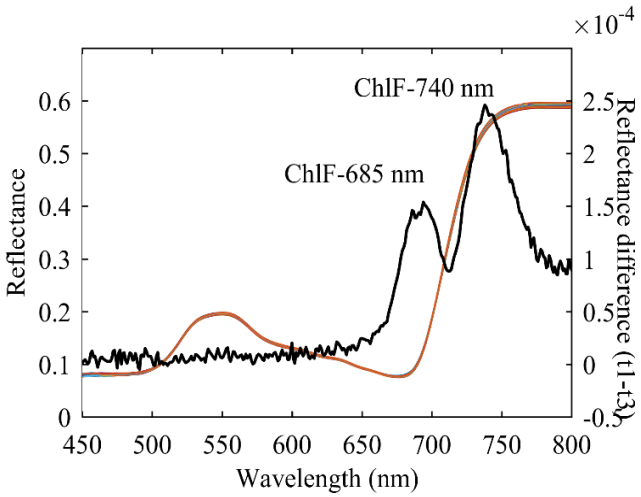


Figure 20 Transient Ra and reflectance difference between Ra at time t_1 and t_3
(For Control Spinach)

For Spinach, the transient of R_a at ChlF emission wavelength showed the same trend as in Corn till first 50 seconds after which it was found to increase gradually (Figure 21). This gradual increases in R_a could be the effect of increasing leaf temperature over the period of measurement like it was found in Corn.

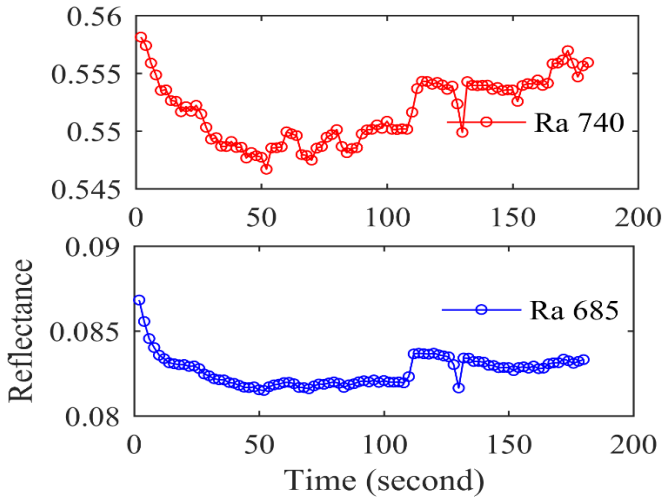


Figure 21 Change in Ra with time at ChlF emission wavelengths
(For control Spinach)

The transient of reflectance deference for spinach leaf shows similar behaviour of Kautsky curve but time taken to achieve steady state in case of spinach was found to be slightly longer (Figure 23) than for Corn (Figure 18). This delay in steady state has been confirmed by simultaneous measurements obtained using PAM (Figure 24).

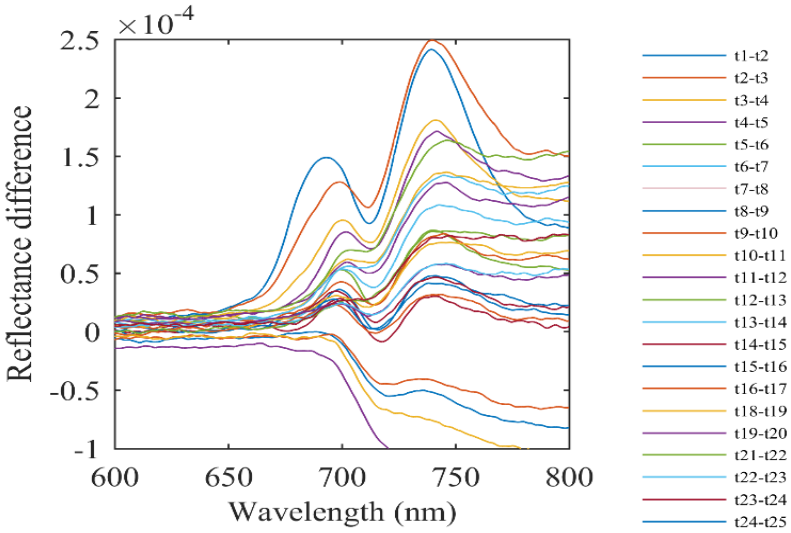


Figure 22 Consecutive reflectance difference in Ra for first 50 seconds
(For control Spinach)

PAM measurements for Spinach shows the slow PSMT phase of induction curve which can also be seen in transient of reflectance difference but appearance of T stage in transient reflectance difference could not be confirmed due to presence of high spectral noise.

The P to S transient decay seen in Figure 19 and Figure 24 has been attributed to re-oxidation of Plastoquinone and the induction of the reversible energy-dependent components of non-photochemical quenching while the further rise i.e. S to M stage has been co-related to the rise in O_2 evolution rate (Stirbet et al., 2014)

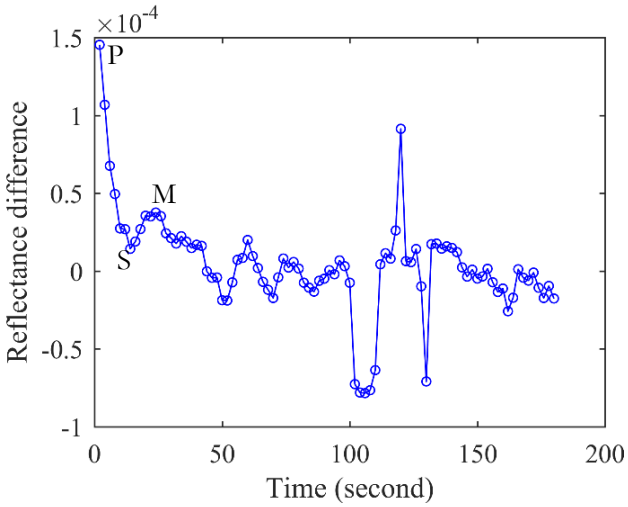


Figure 23 Transient of reflectance difference
(For control Spinach)

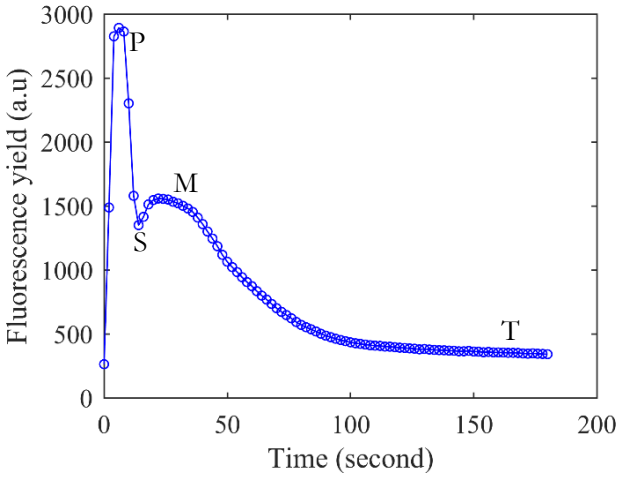


Figure 24 Steady state ChlF measured using PAM
(For control Spinach)

4.3.2. LED lamp as illumination source

The transient change in reflected radiance on sudden exposure of a pre darkened leaf to LED grow light were recorded using ASD. The results of this time resolve study for Corn leaf are presented in Figure 25, from which presence of small ChlF emission peak at 740 nm can be seen in normalized reflected radiance spectra. The time dependent changes in ChlF emission spectrum at 740 nm have also been tracked in these measurements acquired with ASD.

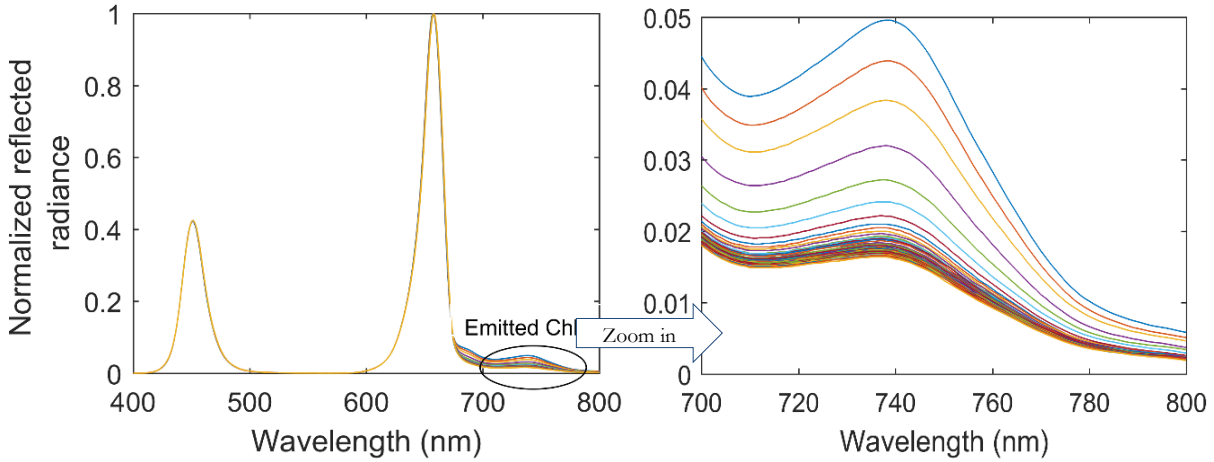


Figure 25 Transient reflected radiance on illumination with LED grow light (For control Corn)

It can be seen from Figure 26 that the transient change in reflected radiance at 740 nm follows the shape of Kautsky curve measured using PAM (Figure 27). The amount of reflected radiance at 740 nm was found to be about 1.5% to 5% of total reflected radiance which is close to the contribution of ChlF to R_a as reported by P. K Entcheva Campbell et al. (2008). The time taken to achieve steady state in case of reflected radiance at 740 nm is similar to that as recorded by PAM measurements (Figure 27).When LED grow light was used, no rise in temperature was recorded by PAM.

On comparing transient reflected radiance at 740 nm for Corn (Figure 26) to Spinach (Figure 29), it is observed that ChlF time decay in Corn is faster than Spinach. Corn ChlF achieves steady state in 50-60 seconds while that in Spinach did not achieve steady state in 3 minutes of measurement period. The amount of emitted ChlF at 740 nm was also found to be slightly higher in Corn than Spinach. As it can be seen from Figure 26 and Figure 29, the time dependent spectral measurements using LED are uniform and do not overlap as seen in results of halogen light.

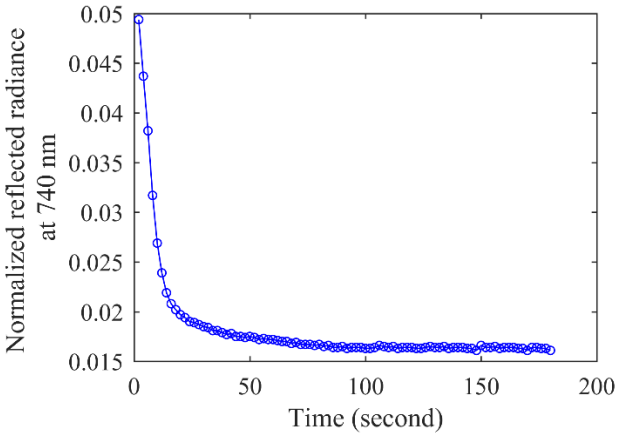


Figure 26 Transient normalized reflected radiance at 740 nm (For control Corn)

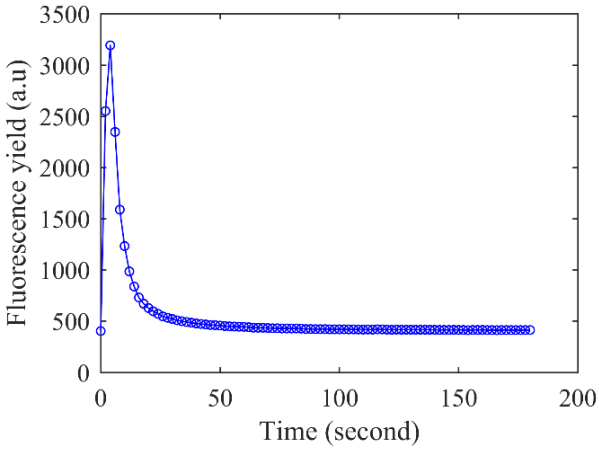


Figure 27 Steady state ChlF measurements using PAM (For control Corn)

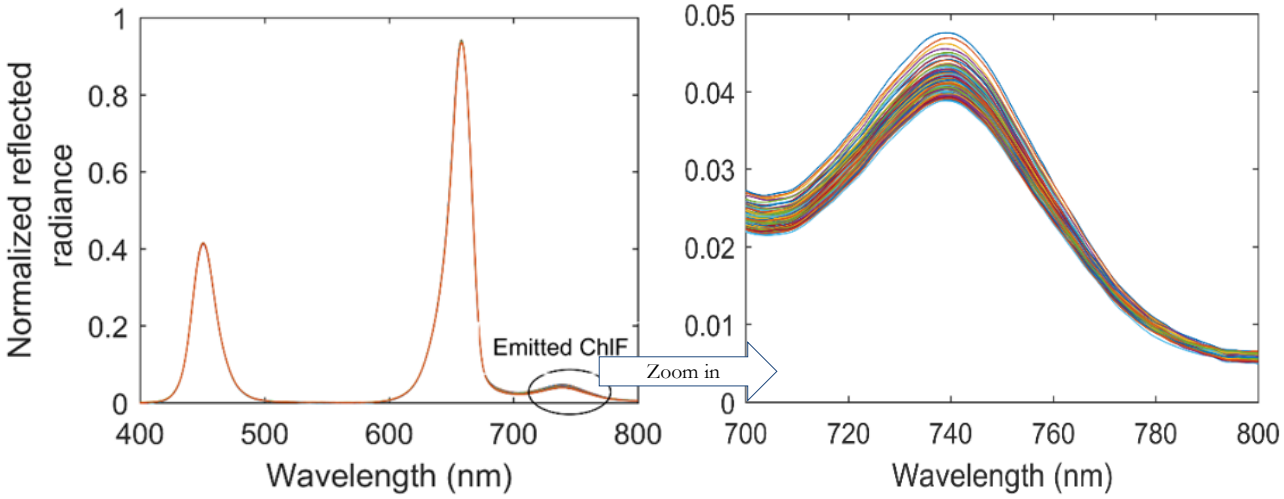


Figure 28 Transient reflected radiance on illumination with LED light (For control Spinach)

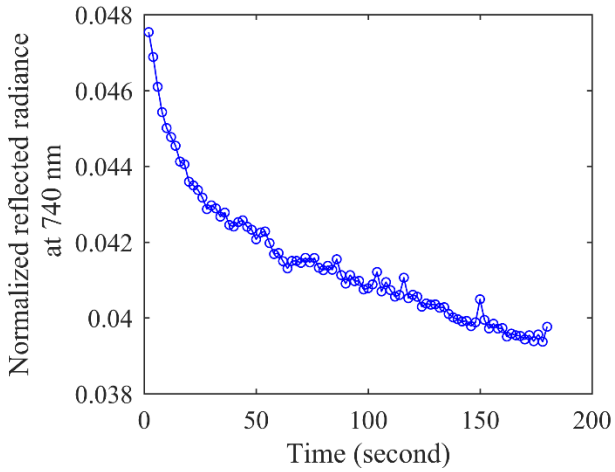


Figure 29 Transient normalized reflected radiance at 740 (For control Spinach)

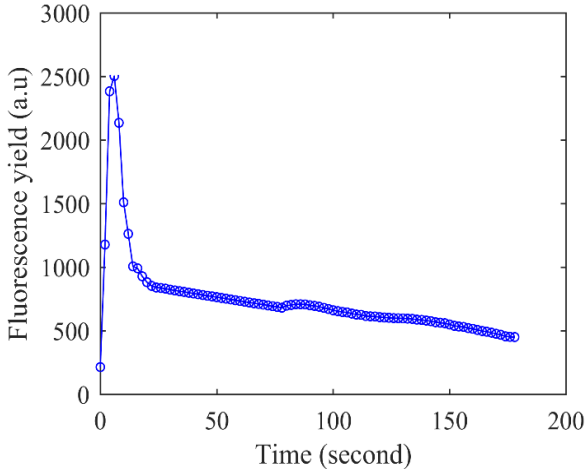


Figure 30 Steady state ChlF measurements using PAM (For control Spinach)

4.4. Experiment 3: To measure the effect of high temperature on the transient change in ChlF on illumination of dark adapted plant.

The dark adapted plant leaf was heated from room temperature to the 40 °C (+/- 5 °C) using hot air blower for 30 minutes and transient reflected radiance spectra on sudden illumination was acquired using ASD. The resulting spectra were observed for effect of temperature on presence of ChlF emission peaks. The results of experiment 3 were compared with results of experiment 2 on the same plant to infer the effect of high temperature on observed ChlF emission.

4.4.1. Halogen light as illumination source

Figure 31shows the transient R_a and reflectance difference for heat treated Corn leaf on sudden exposure to halogen light illumination. It can be seen from this figure that R_a from heat treated Corn leaf increased as compared to non-heat treated Corn leaf (Figure 15).The reflectance difference shows the presence of ChlF in R_a and the ChlF maxima at 685 nm. The height of the ChlF peak at 685 nm has also been increased after the heat treatment as compared to that without treatment. The reason for increasing R_a could be related to the damage caused by heat to the leaf pigments, changed leaf moisture or closing of the stomata which were also been seen in experiment 2.

The cause of the increase in ChlF peak could be the degradation of ChlF pigment and reduction in reabsorption of ChlF at 690 nm. This explanation complies with the fact that the ChlF emission of heat treated Corn leaf was lower than that of non-heat treated corn: thus it was found that the ChlF emission has decreased in heat treated plant. The same observation as of reflectance differences were also recorded by PAM where maximum ChlF yield was found to decrease in heat treated plant. Therefore the possible reason for the decrease in overall ChlF emission and increase in ChlF peak at 685 nm in heat treated plant could be the degradation of chlorophyll-a pigments which in turn affects the reabsorption of ChlF at 685 nm. The heat induced increase in non-photochemical quenching may be another reason for decrease in ChlF emission.

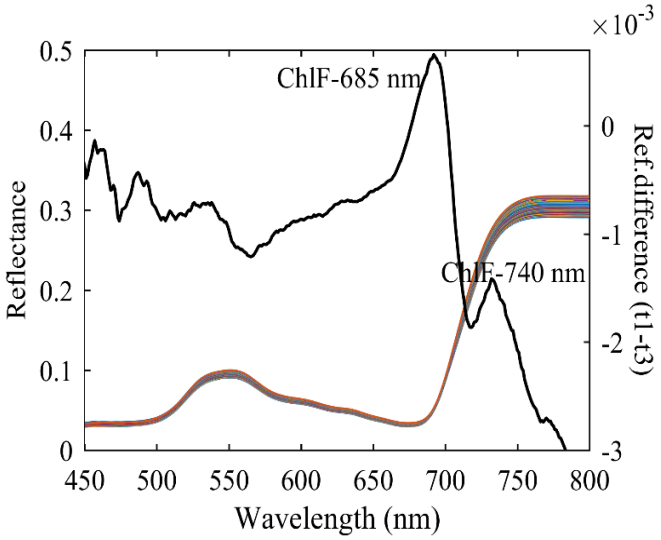


Figure 31 Transient Ra and difference between Ra at t_1-t_3 (For heat treated Corn)

From Figure 32 it can be observed that the Ra at 685 nm increases on sudden exposure to the illumination and slowly decreases for first 10 seconds followed by a fast increase untill the end of 3 minutes measurement, while R_a at 740 nm shows continuous increase from start to the end of the measurement. The increase in R_a at 685 nm and 740 nm could be related to the increasing temperature during measurement which showed direct effect on R_a.

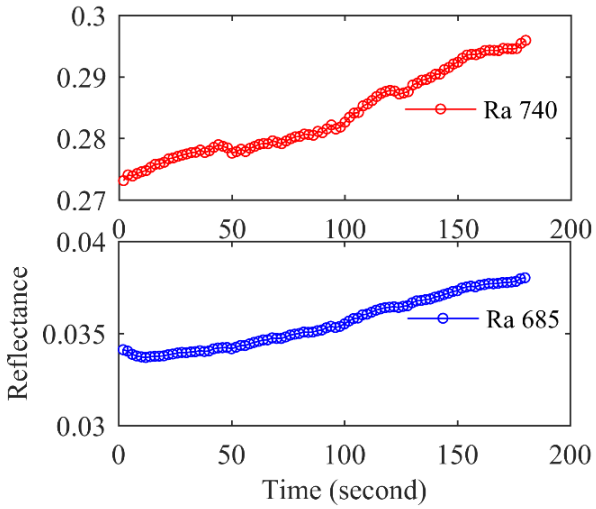


Figure 32 Apparent reflectance at 685 nm and 740 nm (For heat treated Corn)

The time decay in reflectance difference of heat treated plant is shown in Figure 21 and Figure 32. It follows the shape close to the Kautsky curve measured using PAM.

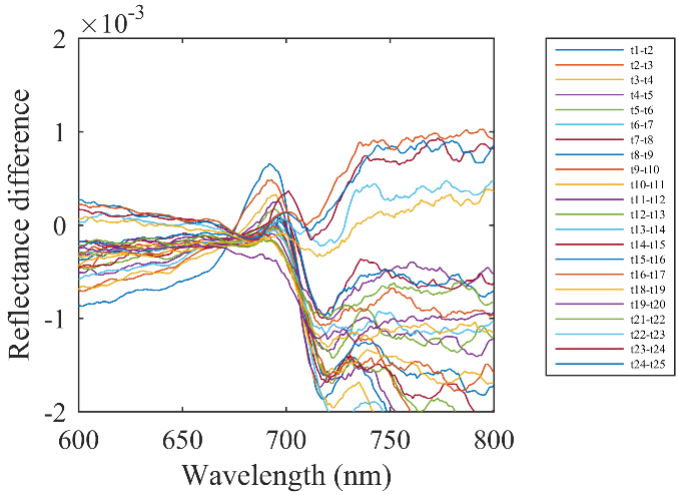


Figure 33 Consecutive reflectance difference in Ra for first 50 seconds (For heat treated Corn)

In the heat treated Corn plant, time taken for achieving steady state ChlF was higher than Corn without heat treatment. The same trend was also seen in the measurements from PAM (Figure 35) which shows a rise in F_0, decrease in F_m and increase in time taken to reach the steady state. The cause of increase in time taken to reach the steady state ChlF could be that the oxidation of reaction centres had slowed down.

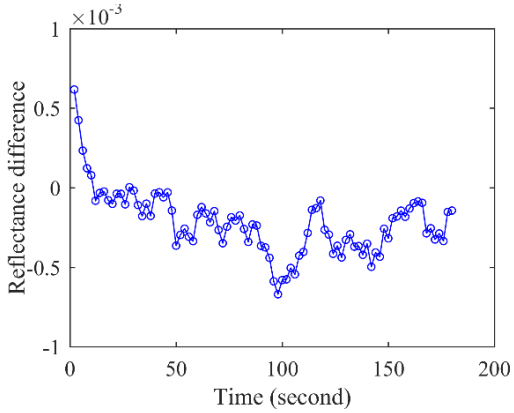


Figure 34 Reflectance difference in Ra
(For heat treated Corn)

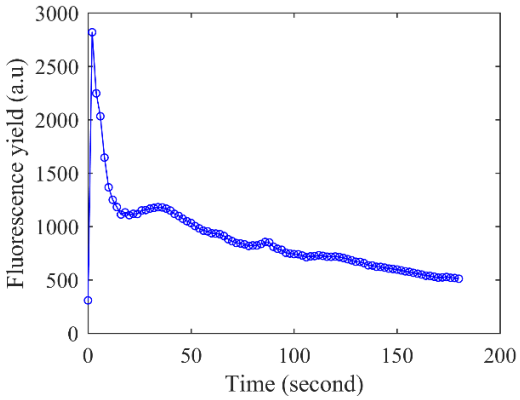


Figure 35 Steady state ChlF measured using PA
(For heat treated Corn)

While looking at the transient reflectance of heat treated Spinach, it can be seen that the NIR reflectance increased as compared to that in non-heated treated Spinach (Figure 20). The reflectance at 685 nm and 740 nm follows the same pattern as in heat treated Corn.

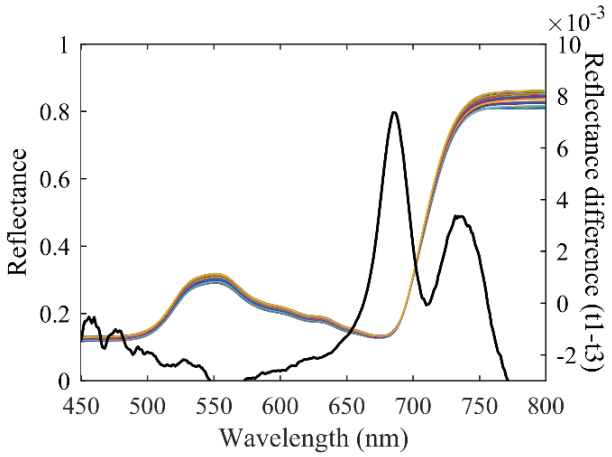


Figure 36.Transient Ra and reflectance difference in Ra at t_1-t_3
(For heat treated Spinach)

The consecutive reflectance difference in heat treated Corn follows the Kautsky curve recorded by PAM but the peak height at 685 nm was found to be higher than peak height recorded in the plant without heat treatment. This increase in peak height at 685 nm was also found in Corn.

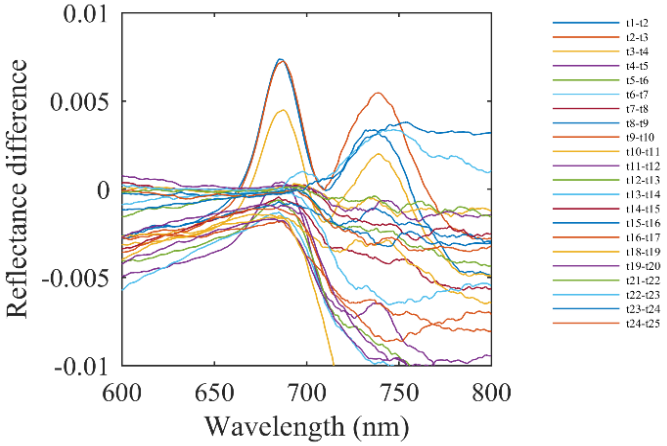


Figure 37 Consecutive reflectance difference in Ra for first 50 seconds (For heat treated Spinach)

The transient of the reflectance difference in Spinach (Figure 39) shows that on sudden illumination, the maximum amount of ChlF emitted at 685 nm increases in heat treated plant compared to the non-heat treated plant and the time taken to reach steady state also increased. The latter observation was also been recorded by PAM. It shows that ChlF at 685 nm on heat treatment follows different patterns in Corn and Spinach.

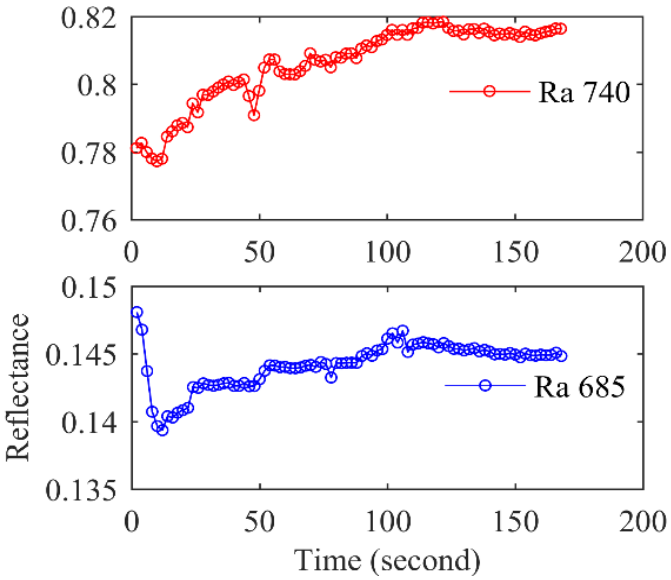


Figure 38 Ra at 685 and 740 nm (For heat treated Spinach)

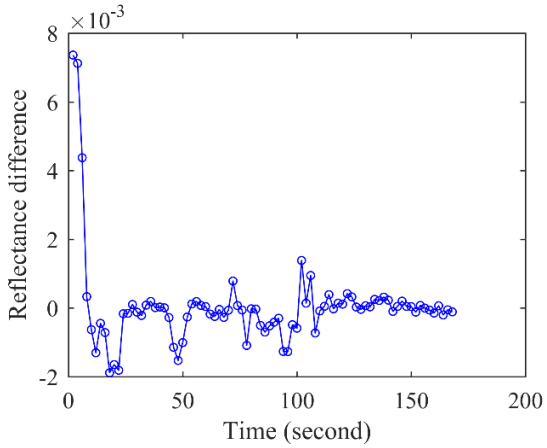


Figure 39 Reflectance difference in Ra at 685 nm (For heat treated Spinach)

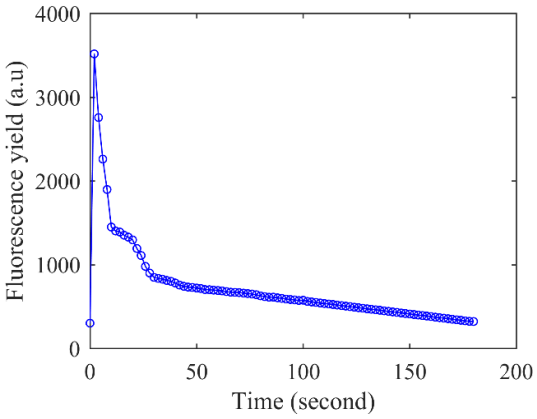


Figure 40 Steady state ChlF measured using PAM (For heat treated Spinach)

4.4.2. LED grow lamp as illumination source

Figure 41 shows the transient reflected radiance for heat treated dark adapted Corn on sudden exposure to illumination from LED grow light. The presence of ChlF emission peak can be seen in this figure at 740 nm. By comparing the result from heat treated Corn (Figure 42) to that of the non-heat treated Corn (Figure 26), it can be seen that the amount of ChlF at 740 nm observed in reflected radiance reduced after heat treatment.

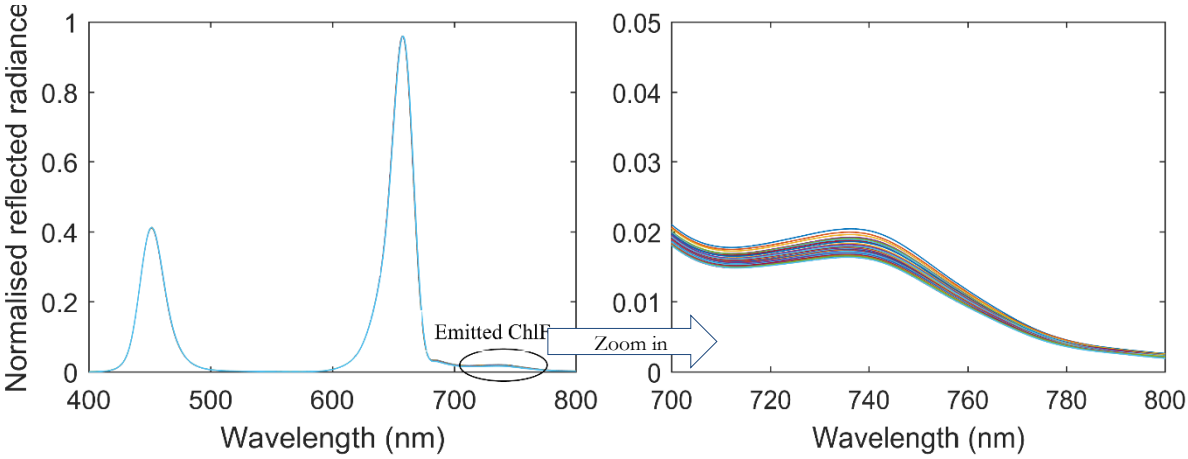


Figure 41 Transient reflected radiance on illumination with LED grow light (For heat treated Corn)

The time taken to achieve steady state also increased in heat treated Corn and these results are in agreement with PAM measurement that show the reduction in ChlF yield after the heat treatment and the delay in achieving steady state ChlF.

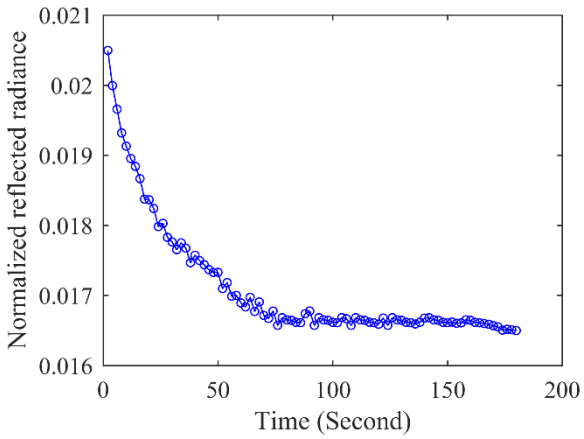


Figure 42 Transient normalized reflected radiance at 740 nm (For heat treated Corn)

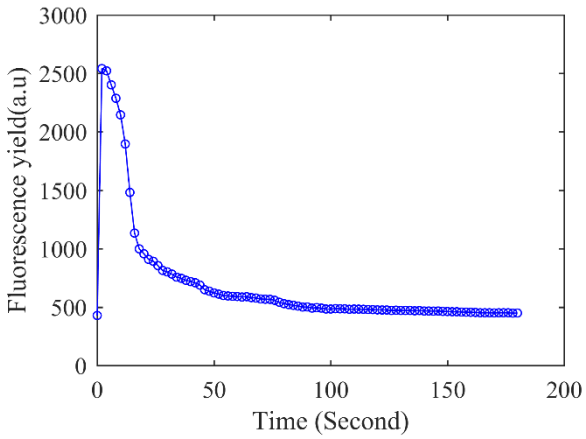


Figure 43 Steady state ChlF measurements using PAM (For heat treated Corn)

The result for Spinach (Figure 44) shows same trend as for Corn where the ChlF emission has decreased in reflected radiance spectra at 740 nm. The time taken to achieve steady state also increased as compared to that of the non-heat treated Spinach leaf (Figure 29).

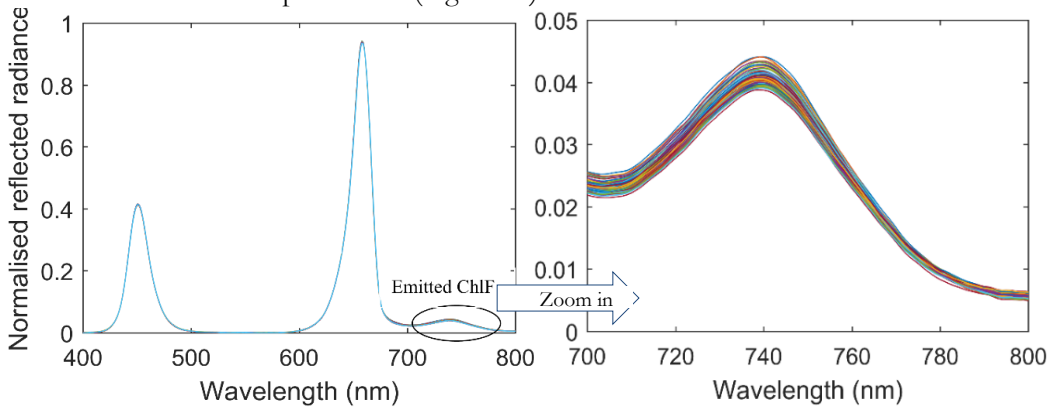


Figure 44 Transient reflected radiance on illumination with LED grow light (For heat treated Spinach)

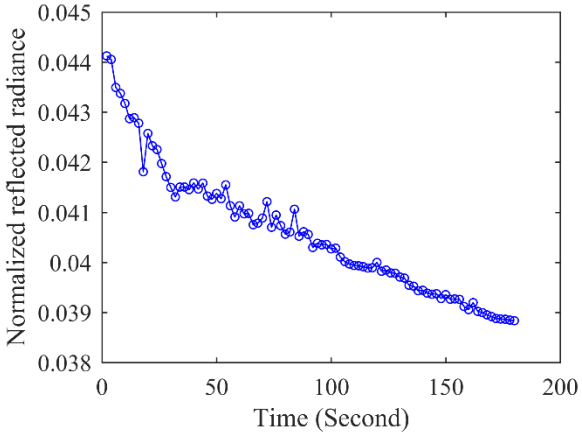


Figure 45 Transient normalized reflected radiance at 740 nm (For heat treated Spinach)

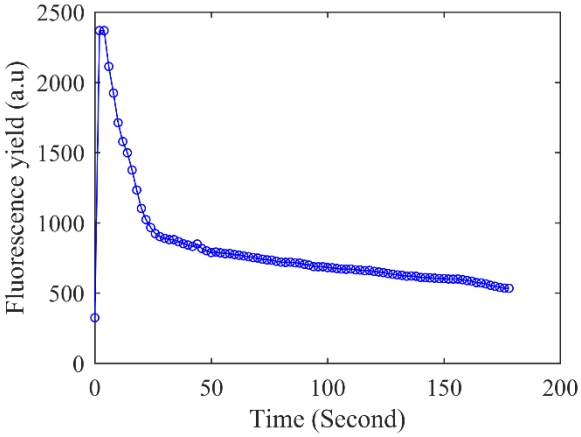


Figure 46 Steady state ChlF measurements using PAM (For heat treated Spinach)

5. CONCLUSIONS

The main objective of the study was to detect the change in ChlF using measurement of low spectral resolution spectrometer and to investigate the effect of high temperature stress on transient change in ChlF on sudden exposer of dark adapted plant. Based on the results obtained in the study, following conclusions were made;

- This study demonstrates that the spectral measurement using ASD can track the change in ChlF excited by halogen and LED light, provided that the signal to noise ratio for ASD is high and the ChlF excitation sources are stable.

  On changing the illumination from low to high intensity, ChlF yield increases depending upon physiological status of the plant.

  The consecutive reflectance differences in a transient R_a when dark adapted leaf was illuminated with halogen light shows the spectrum corresponding to the ChlF emission band. The variation of R_a 685 nm and 740 nm with time after the exposure to light shows similar behaviour to Kautsky curve.

  The transient of reflected radiance at 740 nm when pre-darkened leaf was exposed to LED light shows similar behaviour to the Kautsky curve. Also the reflected radiance at 740 nm shows equivalent proportion to the total reflated radiance as that of ChlF in R_a.

- The heating of the leaf due to illumination source causes significant changes in morphological characteristics of leaf which could result in increase of NIR reflectance. This increase in NIR reflectance may interfere with detection of ChlF when reflectance difference method is used.

- The LED light with blue and red wavelengths is a good source of ChlF excitation and it could be used for leaf level experiments to study the ChlF kinetics.

- The high temperature stress to the C_3 and C_4 plants causes significant changes in functional status of plants. It increases the reflectance due to dehydration of leaf and degradation of pigment molecule.

- High temperature stress decreases the ChlF at 685 nm in Corn while that in Spinach it increases. The ChlF at 740 nm decreases in both, Corn and Spinach.

- Under high temperature stress, time taken to reach the steady state increases in C_3 and C_4 plant.

6. RECOMMENDATIONS

- The present study was performed at leaf level. We recommend to extend this study for canopy level ChlF detection using LED grow light/blue LED light as a ChlF excitation source. Also the application of this method could be performed at green house to detect crop stress where plants are illuminated with blue and red light

- For the detection of ChlF, we assumed that the reflectance factor of leaf does not change but it was found that the reflectance factor could change with time due different stress factors during measurement. We recommend to investigate the time for which reflectance factor remains constant and the consequences of change in reflectance factor on ChlF measurements.

- During this study it was observed that NIR reflectance increases on increase in leaf temperature, and the effect of this increase was also seen on spectral measurements of ChlF, here we recommend to investigate the impact of change in NIR reflectance on the spectral measurements of ChlF.

- It was observed that, spectral measurement of ChlF are susceptible to small variations measurement conditions (e.g. Change in leaf angle) and could become difficult to interpret. Therefore, we recommend to use supplementary information such as leaf temperature, gas exchange, chlorophyll concentration etc. for reasonable interpretation of ChlF signal.

- During literature review, it was found that ChlF yield results are highly depend on measurement protocol and organization of experimental setup. In order to achieve reproducible and comparable measurement, we recommend the standardization of ChlF protocol. We also recommend to use the protocol developed in this study (see Appendix) to detect changes in ChlF using spectrometer at leaf level in laboratory setting.

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APPENDIX 1.

The study recommend to consider the following important points while performing ChlF detection using spectroradimeter. These points could be used as protocol to obtain best results.

1) Sample growth conditions: During this study it has been observed that the plant growth conditions and growth stages play important role in variation of ChlF yield. These variations are mainly due to different physiological responses of plant influenced by environment conditions and developmental stages. Therefore it is recommended to have sufficient knowledge and the record of optimum environmental conditions (e.g. Nutrient, water, temperature, humidity, PAR etc.) necessary for plant growth.

2) Supplementary information: It has been shown by many researchers that ChlF and photosynthesis do not have universal relationship but the relationship varies depending upon species, environmental stresses and their intensity. Therefore it is suggested to collect complementary information such as chlorophyll concentration, leaf temperature, gas exchange etc. which will aid in interpretation of ChlF.

3) Selection of parameter to be measured and pre-treatment of the sample: Selection of ChlF parameters to be measured should be done at the earliest based on the objective of the study which helps in deciding the pretreatment of the sample (Pre-treatments such as dark adaptation of the plant, treatment with DCMU etc.). Ambient conditions of pretreatment may lead to introduction of stress to the sample and this should be avoided by maintaining optimum environmental conditions around the sample.

4) Selection of ChlF excitation source: ChlF emission is wavelength dependent and the selection of appropriate excitation wavelength plays important role in ChlF emission intensity. Therefore the light source should have best suitable wavelength to get expected response of ChlF.

The light source to be used should deliver a stable light output with uniform light coverage over the sample area. Temperature of the Light source should be as minimum as possible so as to reduce the heat stress to plant.
In this case we recommend using LED lights that need less space, generates less heat, are stable and have high efficiency. LED lights are available in selective wavelength ranges which are best suited for ChlF.

5) Spectrometer (ASD): In order to use low spectral resolution spectrometer for ChlF study, the improvement in signal to noise ratio plays important role. Therefore following care should be taken while using ASD which will help improving the signal:

- Calibrate the ASD before start of experiment.
- After switching on the ASD, let it stabilize for 30 minutes prior to making any measurements.
- Optimize the instrument to the response of light source, perform dark current and white reference measurements while optimization.
- Select appropriate integration time according to intensity of your light source.
- Select appropriate spectrum average function based on your need. 25 spectra average is most used function.
- Chose suitable foroptic with necessary field of view. Field of view larger than target area may introduce background reflectance in spectral measurements.

6) Experimental setup and measurement- Organize all components of experiment in scientific and coherent manner such that uniform and repetitive measurements are possible.

Cover the background of the sample (such as experiment platform) with low reflectance material to reduce the background reflectance.

Setup geometric properties of individual measurements. Fix illumination source such that it can illuminate sample uniformly without damaging the sample. Incident angle of 30-45 degree has been reported to be best in many cases

Position ASD fiber optic in such a way that it can capture maximum reflected radiance from sample. For obtaining maximum response at sensor, it is necessary to obtain angular response of spectrum with given source of incident light.

Position the leaf sample using a suitable leaf clip and with minimum geometric distortion of the leaf surface.

Before making a measurement, make sure that the light source is stabilized. Keep the light stable during measurements. Make sure you sample is uniformly illuminated as much as possible. Internal shadows on your sample may cause artifacts in the spectrum.

Perform dark current and white reference measurements per every 10-15 measurements.

Make sure that measurements can be repeated in exactly the same way later.