Laser Induced Chlorophyll Fluorescence of Plant Material

by

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Thesis presented in partial fulfillment of the requirements

for the degree of

Master of Science

at the University of Stellenbosch

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December 2006
Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Signature                        Date
Abstract

Imaging and spectroscopy of laser induced chlorophyll fluorescence (LICF) are emerging as useful tools in plant physiology and agriculture since these methods allow an early detection of plant stress and transformation of plant tissue, before visual symptoms appear. Chlorophyll fluorescence is governed by photosynthetic efficiency and it depends on the plant species and physiological state. In addition, the laser induced fluorescence of chlorophyll molecules in the red and far red spectral range is also used to study basic processes and phenomena in photo-excited molecules. In the work reported here experimental setups used for laser induced chlorophyll fluorescence imaging and spectroscopy techniques were developed to investigate chlorophyll fluorescence under constant illumination and also to detect green-fluorescent protein (GFP) by looking at the chlorophyll fluorescence spectrum and image. He-Ne (wavelength 632 nm), tunable argon ion (wavelength 455 nm), and excimer (wavelength 308 nm) lasers were used as excitation sources. An Ocean Optics spectrometer was used to record the spectrum of the chlorophyll fluorescence and the variation of the chlorophyll fluorescence spectrum with time. The chlorophyll fluorescence spectrum of tobacco leaves expressing GFP was compared to that of control leaves. A charge-coupled device (CCD) camera was used to image the fluorescence from GFP expressing and control tobacco leaves to investigate the effect of GFP genes on chlorophyll fluorescence in relation to the state of the plant material. The spectral analysis technique and image processing procedures were elaborated in order to obtain better information on chlorophyll fluorescence. The results of this work show that the experimental setups and analytical procedures that were devised and used are suitable for laser induced chlorophyll fluorescence analysis. Fluorescence bleaching could be obtained from the time variation of the fluorescence spectrum, and plant expressing GFP can be distinguished from control plants by differences in the laser induced chlorophyll fluorescence.
Opsomming

Die afbeelding en spektroskopiese onderzoek van laser-geënduseerde chlorofil fluoressensie (LICF) word toenemend as nuttige tegnieke in plantfysiologie en die landbouwetenskap gebruik aangesien hierdie metodes vroë waarneming van plant stres en transformasie van plantweefsel toelaat voordat die simptome visueel waarneembaar word. Chlorofil fluoressensie word beïnvloed deur die effektiwiteit van fotosinte wat afhang van die spesie en die fysiologiese toestand van die plant. Verder word die laser-geënduseerde fluoressensie van chlorofil molekules in die rooi en ver-rooi spektraalgebiede gebruik om basiese prosesse en verskynsels in die foto-opwekking van molekules te bestudeer. In die werk waaroor verslag gedoen word is eksperimentele opstellings vir die afbeelding en spektroskopiese onderzoek van laser-geënduseerde chlorofil fluoressensie ontwikkel, met die doel om chlorofil fluoressensie onder konstante beligting te ondersoek asook die teenwoordigheid van die groen fluoresserende proteïen (GFP) waar te neem deur middel van chlorofil fluoressensie spektroskopie en afbeelding. 'n HeNe laser (golflengte 632 nm), 'n afstembare argon-ion laser (golflengte 455 nm), en 'n excimer laser (golflengte 308 nm) is gebruik as opwekkingsbronne. 'n Ocean Optics spektrometer is gebruik om die spektrum van die chlorofil fluoressensie en die tydverandering van die chlorofil fluoressensie te meet. Die chlorofil fluoressensiespektrum van tabakblare waarin GFP uitgedruk is, is vergeëlk met die van kontrole blare. 'n CCD kamera is gebruik om die fluoressensie van die blare waarin GFP uitgedruk is en die van kontrole blare af te beeld sodat die effek van die GFP geen op die chlorofil fluoressensie onderzoek kon word in afhanklikheid van die toestand van die plantmateriaal. Die spektrale analisetegnieke en beeldverwerkingsprosedures is uitgebrey met die doel om beter inligting oor chlorofil fluoressensie te verkry. Die resultate van hierdie werk wys dat die eksperimentele opstellings en analitiese procedures wat ontwikkel en gebruik is geskik is vir die ondersoek van laser-geënduseerde chlorofil fluoressensie. Fluoressensie versadiging kon waargeneem word in die tydverandering van die fluoressensiespektrum, en plante waarin GFP uitgedruk is kon onderskei word van kontrole plante deur middel van verskille in die laser-geënduseerde chlorofil fluoressensie.
Acknowledgements

I would like to thank the following people:

- Prof. H.M. von Bergmann for his excellence, understanding and continuous support in supervising this project.
- Dr. E.G. Rohwer for his guidance, patience and excellence in co-supervision of this project.
- Dr. C.M. Steenkamp for her invaluable input, assistance and efficiency in completion of my work.
- Prof. P.E. Walters for advising and discussing the experimental work.
- Mr. U.G.K. Deutschlander for his help in the technical aspect of the work.
- All my colleagues at the Laser Research Institute, in particular Pieter, Anton, Gibson, Eckhard and Gurthwin for listening to me and providing assistance and support.
- Mrs Rosalie Mfoudou my mother, Elisabeth Okourou my sister and Diana Charlene Lendeme my niece for their moral support and believing that I could achieve this.
- The Gabonese community in Stellenbosch especially Rodolphe Lekogo and Edgard Ngounda.
- My girlfriend, Thembisa Dodo for her love, support and understanding.

My studies were funded by the Gabonese Government.
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Chapter 1

Introduction

1.1 General overview

Laser induced chlorophyll fluorescence is the optical emission from chlorophyll molecules in a plant after absorption of electromagnetic radiation from an active source. The separation of the fluorescence signal from the excitation is a goal to reach in any fluorescence measurement system. This is accomplished by using appropriate optical filters, narrow-band and long-pass filters, which cut off the excitation light and transmit the longer wavelengths of the fluorescence signal or image signal in the case of imaging detector systems. Historically, leaf fluorescence was mainly used as a close contact technique. Nowadays, the development of different forms of lasers has impacted on the method of excitation of the chlorophyll molecules inside leaves, and excitation by repetitive pulse lasers, or continuous wave (CW) lasers, is employed. In recent years laser induced chlorophyll fluorescence (LICF) has been developed as a technique to study the basic processes and phenomena in plant material and used as a remote means of measuring vegetation characteristics such as plant vigour, plant type identification and plant cover estimation [1]. In addition LICF, can be used to make an inference regarding the health and identity of plants. In vivo variable chlorophyll fluorescence has been known for more than half a century, and it has been extensively used by physiologists in the last ten years to characterise the status of vegetation [2, 3]. The development and use of different types of laser sources for excitation of chlorophyll molecules, such as CW and pulsed lasers have made chlorophyll fluorescence a very popular tool in plant physiology, environmental studies and
agriculture.

Studies on chlorophyll fluorescence emission spectra and photosynthetic apparatus have indicated an inverse proportional relationship between fluorescence intensity and photosynthetic process performance [1]. Therefore, chlorophyll fluorescence emission of dark-adapted leaves can provide considerable information on the organisation and function of the photosynthetic apparatus. With the development of high temporal and spatial resolution detector systems, the use of the chlorophyll fluorescence signal as probe of the photosynthetic apparatus and stress levels in plant material has become a routine technique in many laboratories and industries. In the environment and the field of plant physiology, chlorophyll fluorescence is used to study pollution by means of biomonitoring of the plant, the photosynthesis process and the physiological stress of the plant. Environmental factors, such as excessive irradiation, heat, or water stress, either directly or indirectly affect the photosynthetic function of leaves and the structure of the photosynthetic apparatus. In addition, they often modify the optical and fluorescence properties of leaves [4, 5, 6]. So various types of chlorophyll signatures have been applied with the view to investigate and describe the photosynthetic processes and to detect stress and strain in the photosynthetic apparatus. In agriculture, chlorophyll fluorescence is used to monitor growth factors such as hormones, herbicides and fertilizers, and the effect of drought, heat, light and salt on plants. It is also used to detect the infection and damage caused by pathogens on plant material, for quality control of the freshness, taste, colour and consistency of vegetables and fruits, and to determine the effect of storage on vegetables, fruit and flowers [7].

In addition, the chlorophyll fluorescence kinetics of leaves, known as the Kautsky effect, has been used to study the functioning of plants. It provides information not only about the photochemical and non-photochemical quenching coefficients but also information that can be used to determine the chlorophyll fluorescence ratio (red/far red), which is an indicator of the status of the plant, and also the in vivo chlorophyll content [8]. These parameters are used to detect the malfunction and damage of the photosynthetic apparatus and its function.

The chlorophyll fluorescence spectrum of plant material is characterised by two bands, red and far red, which have their maxima at 690 nm and 730 nm respectively. The deficiency in certain nutrients required for the growth of plant material is manifested by changes in laser induced chlorophyll fluorescence intensities at these specific wavelengths. The presence of certain
protein markers and heavy metals in plant material can impair the functioning of a number of physiological factors, including various enzymes, protein synthesis, chloroplast structure and function, and photosynthesis pathways, which can be detected by chlorophyll fluorescence. For instance, toxic levels of Zn have been shown to inhibit photosynthesis [9]. Therefore early stress detection in plants, before visual damage symptoms are noticeable, is required in order to reactivate the plant’s vitality by suitable countermeasures.

The ability to non-destructively visualise transient and stable gene expression has made green fluorescent protein (GFP) a most efficient reporter gene for routine plant transformation studies. The question that one may ask is: does the presence of the GFP have an impact on the photosynthesis process; consequently, does GFP influence the chlorophyll fluorescence?

1.2 Goals

The primary goal of this study is to develop simple and suitable experimental setups for the recording of chlorophyll fluorescence. The second goal is to test these experimental setups and evaluate different methods and techniques of analysing chlorophyll fluorescence spectra and imaging. The third goal is to study the effect of GFP on chlorophyll fluorescence, notably on photosynthesis process.

1.3 Layout of thesis

The general overview of the concepts, methods and use of laser induced chlorophyll fluorescence in the role of photosynthetic activity are briefly outlined in Chapter 1.

In Chapter 2 the background of laser induced chlorophyll fluorescence is presented. First the basics of fluorescence are described, including not only the basic processes that lead to fluorescence but also the shift between the absorption and the emission spectra, namely the Stokes shift. Second, an overview of chlorophyll fluorescence of green plant material is given.

In Chapter 3, the setups used for fluorescence spectroscopy, using CW lasers as excitation sources, and setups used for fluorescence imaging, using pulse and CW lasers are described. The red/far red chlorophyll fluorescence ratio is used as a parameter to analyse chlorophyll fluorescence spectra and images obtained. In addition, the methods used for the analysis of
spectra and the imaging processing technique are described. In order to make a better analysis of the fluorescence signal, signal processing is used in the deconvolution of the signal, or in the cleaning of images.

In Chapter 4 the results of investigations of the GFP expression in plant tissue by means of chlorophyll fluorescence are presented. Two methods were used to make the investigation. The first method is the spectroscopic analysis of chlorophyll fluorescence spectra using the fluorescence measurement at single leaf spots, namely the single point measurement. The second method is leaf fluorescence imaging.

In Chapter 5 a summary of results and conclusions are presented, and recommendations for further study are given.
Chapter 2

Background

Fluorescence is a luminescence process in which molecules emit light from electronically excited states created by either a physical, mechanical, or chemical mechanism. Generation of luminescence through excitation of a molecule by ultraviolet or visible light photons is a phenomenon termed photoluminescence, which is formally divided into two categories, fluorescence and phosphorescence, depending upon the electronic configuration of the excited state and the emission pathway. In fluorescence, electron de-excitation occurs almost instantly, and emission from a fluorescent substance ceases when the exciting source is removed. Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and to re-emit light of longer wavelength after a brief interval.

2.1 Fluorescence

A molecule exposed to an electromagnetic field absorbs energy in discrete amounts, named quanta, if both the resonance condition and the selection rules of quantum mechanics transition are satisfied. Likewise, emission of a photon through fluorescence or phosphorescence is also measured in terms of quanta. The energy in a quantum (Planck’s Law) is expressed by the equation:

$$\Delta E = E_i - E_j = h\nu = h\frac{c}{\lambda}$$  \hspace{1cm} (2.1)

where $E_i$ and $E_j$ are the energies of the two states that participate in the transition, $\nu$ and $\lambda$.
are the frequency and wavelength of the incoming photon respectively, \( h \) is Planck’s constant, and \( c \) is the speed of light.

When materials absorb light, molecules are taken to the excited state. Then at that state two processes may occur: radiative processes, where a photon is emitted to bring the molecule back to the ground state, and nonradiative processes, where either the excited state energy is dissipated as heat or the energy absorbed is captured by the material to produce chemical reactions called photochemical processes [10]. In this section the study is limited to the radiative process: fluorescence.

Fluorescence is the phenomenon in which absorption of light of a given wavelength by a fluorescent molecule is followed by the emission of light at longer wavelengths. When atoms or molecules are irradiated by light of a wavelength that corresponds to the energy of an allowed transition from the ground state to the excited state, there will be an absorption of photons which is followed by the release of radiation, causing the atoms or molecules to return to the original level. The magnitude of the fluorescence intensity released depends on both intrinsic properties of atoms or molecules and on controlled experimental parameters, including the intensity of the absorbed light and the concentration of the chlorophore. The intensity of the emitted light, \( F \), is described by the relationship:

\[
F = \phi I_0 (1 - e^{\varepsilon b c})
\]  

(2.2)

where \( \phi \) is the quantum efficiency of the combined absorption and fluorescence process, \( I_0 \) is the incident radiation power, \( \varepsilon \) is the molar absorptivity, \( b \) is the path length of the cell and \( c \) is the molar concentration of the fluorescent molecule.

The fluorescence process is governed by three important events. The first is the electronic excitation of a molecule by an incoming photon, which happens in femtoseconds, this is followed by vibrational relaxation within the excited state, bringing the molecule to the lowest vibrational level of the excited state. This phenomenon can be measured in picoseconds. The final process is the emission of a longer wavelength photon and return of the molecule to the ground state, which occurs in a relatively long time period of nanoseconds.

When an atom or molecule is excited by light into a high vibronic state, there exist multiple ways for relaxation, such as the internal conversion (IC), and intersystem crossing (ISC). The
fastest process is internal conversion, where electronic energy is converted into vibrational energy. Suppose the excitation is to the electronic level $S_2$, then a nonradiative crossing is generated between two electronic states of the same spin multiplicity, from the singlet state $S_2$ to the state $S_1$, as illustrated in Figure 2-1. This IC process is followed by a fast vibrational relaxation, where the excess vibrational energy is dissipated into heat, in which the molecule exchanges vibrational quanta with its environment. Note that during this relaxation process fluorescence may occur, but the total amount of fluorescence observed from these higher vibronic states is very low and difficult to detect. Then the molecule ends up at the lowest vibrational level of the electronic state $S_1$. From here it will return to the singlet ground electronic state $S_0$ by emitting a photon (fluorescence). Alternatively, there is another radiationless deactivation pathway of the singlet state $S_1$ to the triplet state $T_2$, as indicated in Figure 2-1. This is called intersystem crossing. Transitions from a singlet state to the triplet state are quantum mechanically forbidden by spin selection rules but under certain conditions such as strong spin-orbital coupling, these transitions may take place. The various energy levels involved in the absorption and emission of light by a fluorophore are classically represented by a Jablonski energy diagram, as shown in Figure 2-1. The horizontal black lines represent electronic energy levels and the horizontal grey lines the various vibrational energy states. Radiative and non-radiative transitions between the states are illustrated as straight and wavy arrows respectively (see Figure 2-1).

For fluorescent molecules, the decay via internal conversion from the lowest excited singlet state to the ground state is slow. The main reason is the large energy gap between $S_1$ and $S_0$. For instance, for chlorophyll-a the fluorescence lifetime of $S_1$ is a few nanoseconds.

The absorption of a photon always occurs from the lowest ground state level to one of the higher vibronic states. The emission occurs from the lowest excited state to one of the higher vibronic ground states. Consequently fluorescence occurs always at lower energies than absorption, and thus is red-shifted. This shift occurs between the peak of the absorption band and that of the fluorescence band. In addition, since fluorescence is a slow process, the environment surrounding the excited molecule may cause it to relax non-radiatively and this will give rise to a further red-shift of the fluorescence. This phenomenon, fluorescence red-shifted from the absorption, is called the Stokes shift, (see Figure 2-2). The amount of Stokes shift is a
Figure 2-1: Jablonski diagram showing the electronic and vibrational states and levels [36].
measure of the relaxation process occurring in the excited state, populated by absorption. The Stokes shift may come from an environmental effect and also from a change of the geometry of the emitting excited state. This shift is caused by the loss of energy. This can be seen from the difference between the energy of the absorbed photon and that of the emitted photon. The Stokes shift is measured as the difference between the central wavelengths in the excitation and emission spectra of a particular fluorochrome or fluorophore. The size of the shift varies with molecular structure, but can range from just a few nanometers to over several hundred nanometers. For example, the Stokes shift for fluorescein is approximately 20 nanometers, while the shift for quinine is 110 nanometers [11].

The existence of the Stokes shift is critical to the extremely high sensitivity of fluorescence imaging measurements. The red emission shift enables the use of precision bandwidth optical filters to effectively block excitation light from reaching the detector so the relatively faint fluorescence signals (having a low number of emitted photons) can be observed against a low-noise background.

Careful analysis of the fluorescence emission spectrum may reveal several important features. The emission spectrum is independent of the excitation energy (wavelength) because of rapid internal conversion from higher initial excited states to the lowest vibrational energy level of the $S_1$ excited state. For many of the common fluorophores, the vibrational energy level spacing is similar for the ground and excited states, which results in a fluorescence spectrum resembling the absorption spectrum. This is due to the fact that the same transitions are most favorable for both absorption and emission. Because emission of a photon often leaves the fluorophore in a higher vibrational ground state, the emission spectrum is typically a mirror image of the absorption spectrum. In effect, the probability of an electron returning to a particular vibrational energy level in the ground state is similar to the probability of that electron’s position in the ground state before excitation. This concept is known as the Mirror Image Rule. The resulting emission spectrum is a mirror image of the absorption spectrum displayed by the hypothetical chromophore.

Electronic fluorescence spectroscopy allows us to carry out a multiparameter analysis using its emission spectra, absorption spectra and the lifetime of emission.

Three fundamental parameters commonly used in describing fluorescence phenomena are the
Figure 2-2: The shift between the absorption spectrum and the emission spectrum: Stokes shift[37].
extinction coefficient, quantum yield and fluorescence lifetime. The most important parameter here is the quantum yield.

Quantum yield (sometimes incorrectly termed quantum efficiency) is a gauge for measuring the efficiency of fluorescence emission relative to all of the possible pathways for relaxation, and is generally expressed as the (dimensionless) ratio of photons emitted to the number of photons absorbed. In other words, the quantum yield represents the probability that a given excited fluorochrome will produce an emitted photon (fluorescence). Fluorescent molecules commonly employed as probes in microscopy have quantum yields ranging from very low (0.05 or less) to almost unity (the brightest fluorophores). In general, a high quantum yield is desirable in most imaging applications.

### 2.2 Chlorophyll Fluorescence

Chlorophyll is the molecule that captures the sunlight energy and is called a photoreceptor. It is found in the chloroplasts of green plants, and is what gives plants their green colour. Although there are many different pigments in green leaves, the most important in the absorption of light are the chlorophylls. There are two types of chlorophyll, named chlorophyll-a and chlorophyll-b. They differ only slightly in the composition of one of their sidechain substituents (in chlorophyll-a it is a -CH3 and in chlorophyll-b it is a -CHO), see Figure 2-3. Both of these two chlorophylls are very effective photoreceptors because they contain a network of alternating single and double bonds.

When sunlight strikes the plant leaves, chlorophyll molecules absorb it. That energy can undergo one of the three processes: it can be used to initiate the process of photosynthesis and then the production of biomass (photochemical process), the excess energy can be dissipated as heat, or it can be re-emitted as light (chlorophyll fluorescence). Figure 2-4 summarises the three processes. There are two photosystem complexes that are involved in the photochemical process: photosystem I (PSI) and photosystem II (PSII). Both photosystems contain the two chlorophyll pigments (chlorophyll-a and -b) that are involved in the photosynthetic mechanism. In the chloroplasts the antenna molecules (chlorophyll-a and -b) are tuned to absorb light of a particular wavelength. The antennae transfer the energy that they absorb to the reaction center
Photosynthesis activities of green plants depend on the chlorophyll content of their leaves. In photosynthesis, chlorophyll acts as a photosynthesiser. The level of fluorescence emitted by plants upon exposure to light is governed by photosynthetic efficiency. The study of in vivo chlorophyll fluorescence (ChlF) of green plants is highly correlated to knowledge of the mechanism of photosynthesis. It provides basic information on the functioning of the photosynthetic apparatus and on the capacity and performance of photosynthesis. Chlorophyll fluorescence is also used to indicate and evaluate stress levels or physiological damage of plant, such as photo-inhibition, deficiency of water and nutrients, damage caused by pollution, temperature, and diseases. In addition, the ratio of the red to far red fluorescence is a good indicator of stress and chlorophyll content.

Chlorophyll absorbs both the longer and the shorter waves in the visible spectrum. There
Figure 2-4: A representative diagram of the three processes that occur in photosystems PS II and PS I of green leaves when they absorb energy: the photochemical process, fluorescence and heat dissipation [35].
are two main excited states available in chlorophyll-a, resulting in the absorption of blue and red light. The red absorbed light raises the molecule to the lowest, and most important of, the excited levels, $S_1$, while blue light excites the molecules to the third excited state, $S_3$. The chlorophyll fluorescence emission spectrum depends on the type of chloroplast, which contains the chlorophyll, age of the plant material, physiological state of the leaf and the intensity of the light. The chlorophyll fluorescence of green plants at room temperature possesses a typical double peak, with two distinct maxima at about 690 nm (red) and 730 nm (far red) (Figure 2-5). The fluorescence of the red band and far red band are provided by the PS II while the PS I has only a small contribution to the fluorescence of the far red band [12, 13, 14]. Figure 2-5B exhibits the spectrum of chlorophyll fluorescence with these two peaks. Chlorophyll-a is the only fluorophore species present in leaves that is responsible for the emission of fluorescence in this region of the spectrum, while chlorophyll-b transfers all its excited energy to chlorophyll-a in vivo. Chlorophyll-b, in chloroplasts, fluoresces only when they are isolated, but in leaves only the fluorescence of chlorophyll-a can be detected. Even when a mixture of chlorophyll-a and chlorophyll-b is illuminated at a wavelength that stimulates only chlorophyll-b (absorption wavelength around 470 nm and 650 nm), only the chlorophyll-a fluorescence will be detected. In fact, the energy absorbed by chlorophyll-b is automatically transferred to chlorophyll-a which will be excited [15].

As indicated in Figure 2-5A, the absorption spectrum of chlorophyll-a largely overlaps the 690 nm fluorescence band, therefore at 690 nm the chlorophyll fluorescence undergoes reabsorption. Consequently, the shape of the relative height of the chlorophyll fluorescence emission band will suffer severe changes. These changes depend not only upon the chlorophyll content of the leaf but also on the relative concentrations of chlorophyll-a and b. At low concentration of chlorophyll-a, the red fluorescence is greater than the far red fluorescence. With increasing chlorophyll content there is a decrease of the red fluorescence peak and an increase of the far red peak.

The shape of the chlorophyll fluorescence spectra and the value of the ratio of the fluorescence intensities at the two maxima are related to the chlorophyll content of the leaf. A very low chlorophyll content in leaves produces a chlorophyll fluorescence spectrum with only one maximum of the red band and a very slight shoulder of the far red band. When the quantity
Figure 2-5: Illustration of the reabsorption effect of chlorophyll fluorescence inside leaves. Shown by the dashed line is the real Chlorophyll-a fluorescence recorded in chlorophyll extract from the leaf [35].
of chlorophyll is increased, the fluorescence of the red band, at 690 nm, decreases considerably due to re-absorption of the emitting fluorescence by the chlorophyll. Under these conditions the value of the ratio of red to far red band will undergo a significant change. In fact these changes show that the ratio is an indicator of the chlorophyll content. An increase of the $F_{690}/F_{730}$ peak intensity ratio is not only indicative of a lower chlorophyll content; the values also increase when the process of photosynthetic quantum conversion is affected and declines. The intensity ratio of red to far-red chlorophyll fluorescence is inversely related to the photosynthesis activities. When photosynthesis decreases because of various stress activities, the $F_{690}/F_{730}$ chlorophyll fluorescence intensity ratio increases [1]. In addition, the fluorescence intensity ratio in intact leaves is strongly influenced by changes of certain environmental factors, such as a temperature variation [14]. The ratio of the two bands also serves to indicate the presence of invasion of strange proteins and the deficiency of certain nutrients inside the plant [16].

Besides the chlorophyll fluorescence spectroscopy technique, which gives good spectral resolution, chlorophyll fluorescence imaging has taken over from the spectral technique. It provides spatially resolved fluorescence information on all parts of the irradiated leaf. For instance, when a leaf is irradiated, the chlorophyll fluorescence over the whole leaf can be discontinuous. Therefore certain parts of the leaf can have a high, other a medium, and other parts a very low chlorophyll fluorescence yield [17]. This shows that the photosynthetic activity is not evenly distributed across the area. In addition chlorophyll fluorescence imaging has been used for the detection of various types of strain and stress. For example, when the leaves are treated with the herbicide Diuron (DCMU), which is known to efficiently block the photosystem II, chlorophyll fluorescence imaging shows an increase of red and far red fluorescence compared to the non-treated leaves. Chlorophyll fluorescence imaging shows also a certain leaf patchiness indicating the presence of Diuron in leaves, and the inhibition of photosynthetic electron transport [8].

The intensity of chlorophyll fluorescence changes as a function of time. This change is known as the Kautsky effect. It occurs when a leaf is suddenly illuminated at a high irradiation level, causing a very rapid increase in the chlorophyll fluorescence yield, that reaches a maximum within 100 to 200 milliseconds. After the chlorophyll fluorescence reaches its maximum, the red and far red chlorophyll bands decrease exponentially, with different time constants [12]. The
rise of the chlorophyll fluorescence is a consequence of the reduction of electron acceptors in the photosynthetic pathway. When PSII absorbs light and the electron acceptor has accepted one electron, this acceptor may not be able to accept another electron until it has passed that electron to the subsequent electron carrier. So, at this point, the reaction centres of PSII are closed. This closing of the reaction centres of PS II will cause a temporary reduction in the flow of energy into the photochemistry chain and therefore an increase of the chlorophyll fluorescence. After reaching the maximum there is a slow decline of chlorophyll fluorescence to a steady-state level that is reached between 2 and 5 minutes. This is due to an increase of the photochemical and non-photochemical quenching [18]. The increase of the photochemical quenching is due to light-induced activation of the enzymes which participate in the photochemical process and the opening of stomata. Therefore this activation will increase the photosynthetic activity. The increase of non-photochemical quenching originates in the increase of the efficiency with which energy is converted to heat.

In order to understand photochemical processes in promoting photosynthetic activities better, various chemicals are used on the plant to either cause stress or enhance fluorescence emission of the plant. One such chemical is Green Fluorescence Protein (GFP).

GFP is a fluorescent protein derived from the photogenic cell of the jellyfish Aequorea. This protein is used as a marker. It is often used as an early reporter of plant transformation. In its native form it absorbs at 395 nm and 475 nm, with a maximum fluorescence around 508 nm. Generally the GFP is introduced into the plant cell as a GFP gene which will synthesise the GFP. The GFP gene can undergo several mutations. The mutagenesis of the primary sequence gives a wide variety of GFPs with broad spectral properties. Those mutations produce different forms of fluorophores. They have different excitation and emission wavelengths as indicated in Figure 2-6: BFP (382/446 nm), CGP (434/476 nm), GFP (475/504 nm), EGFP (488/509 nm), YFP (514/527 nm), RFP (558/583 nm). The most convenient excitation sources are Ar-UV ion laser (351, 364 nm), Ar laser ion (457 nm, 488 nm, 514 nm), green He-Ne laser (543 nm), Hg lamp, Ti:sapphire laser (it was found that these GFPs could be excited by two-photon excitation (TPE) when excited with 800 nm of the Ti:Sapphire laser TPE). The GFP and those mutant fluorophores are used mostly for bioimaging purposes. The localisation of the absorbing, and consequently the fluorescing, part of the fluorophore unit may protect it from
the effect of the bulk solvent to impacting on some characteristics of the GFP. GFP is a robust protein; it is resistant to denaturation by heat (it denatures at temperatures above 90°C and pH values outside of the range 4-12), it can be coupled to another protein, it is a noninvasive fluorescent marker for living cells, and has wide range of applications, such as a cell lineage tracer, a reporter of gene expression or a measure of protein-protein interaction [10].

Green fluorescence protein (GFP) is a protein which fluoresces in the green portion of the visible spectrum. It has become a useful tool for making expressed proteins fluorescent, by introducing its genes into the plant. It is known as a noninvasive, novel and simple marker, and is used as an in vivo marker in a wide variety of biological systems. GFP can be used as a visual and non-destructive reporter; it offers the opportunity to follow the growth and development of transformed cells. When GFP genes are inserted into a plant which has a significant amount of chlorophyll, the green GFP fluorescence can, to some extent be masked by the red chlorophyll fluorescence, and it can be difficult to detect the GFP fluorescence. So the question that one may ask is: does GFP has an influence on the photosynthetic process, and therefore on chlorophyll fluorescence?
Figure 2-6: The diagram showing the excitation and emission spectra of different protein derived from the GFP: BFP, CFP, GFP, YFP. (Source: Clonetech Laboratories Inc., Palo Alto, Calif.)
Chapter 3

Experimental Techniques and Setup

3.1 Fluorescence Spectroscopy

3.1.1 Setup

Spectroscopy measurements of chlorophyll fluorescence of plant material were performed by irradiating the leaves with two different sources of excitation in the same experimental configurations. The first source used was a He-Ne laser, which has a visible wavelength of 632.8 nm and produces a typical output power of 5 mW. The second source used, in the same configuration, was a line tunable CW argon ion (Ar$^+$) laser operating at a wavelength of 455 nm. The output power of the argon ion laser was first fixed at 5 mW and then at 10 mW.

A leaf of a potted tobacco plant was placed at a distance of between 1 m and 2 m from the laser. The setup is illustrated in Figure 3-1. Long-pass filters (a RG645 filter for the He-Ne laser was used to block light at wavelengths shorter than 645 nm, and a 550 filter for the Ar$^+$ laser was used to block light at wavelengths shorter than 550 nm) were placed behind the leaf to cut off the scattering from the laser in order to record only the fluorescence. A fibre optic of 200 µm diameter and 1 meter length was used to guide the collected fluorescence light to a high-resolution Ocean Optics spectrometer (HP4000 spectrometer). The Ocean Optics spectrometer displayed the fluorescence spectrum in the range from 200 to 1100 nm.

The acquisition of the spectrum was done in transmission and hence the leaf had to be as close as possible to the filter to enable the maximum intensity of the fluorescence to be coupled
Figure 3-1: Spectroscopy setup: Chlorophyll fluorescence setup using a He-Ne or argon ion laser as sources of excitation of the chlorophyll molecule in tobacco leaf, and determination of chlorophyll fluorescence.

The shape of the fluorescence spectrum may undergo significant changes depending on the experimental procedure. The intensity of the fluorescence depends on the concentration of the emitting chlorophyll. It also depends to some extent on the optical properties of the leaf, which determine the penetration of the excitation radiation into the tissue [19]. Additionally, while the experiment was being carried out, it was noticed that the configuration of the leaf and the distance between the leaf and the optic fiber (as shown on the figure 3-1) had a great influence on the intensity of the fluorescence. In order to obtain the maximum intensity of chlorophyll...
fluorescence, the leaf was brought as close as possible to the filter, and was actually placed in
direct contact with the filter.

3.1.2 Spectral analysis techniques

The use of the Ocean Optics software allows the recording of spectra of the chlorophyll fluores-
cence and saving the data to a text file. Since Ocean Optics software does not permit analysis of
the spectra, the data were imported into a dedicated analysis software (Microcal Origin version
5.0) for spectral analysis. This software uses the default import settings specified in the ASCII
Import Options to import the data file. The graph of the chlorophyll fluorescence spectrum
can then be reconstructed.

Once the spectrum was plotted, a Gaussian function curve fitting was performed on the
peaks of the spectrum. The green curves exhibited in Figure 3-2 represent the two Gaussian
fits. Making the summation of the two Gaussian spectral peaks, one ends up fitting the whole
recorded spectrum. The red curve in Figure 3-2 indicates the result of the fitting. The Gaussian
spectral function for the curves fitting was done in the region between 620 nm and 850 nm.
Each of these Gaussian peak fit curves is characterised by the equation:

\[
y = y_0 + \frac{A}{\sqrt{\pi w^2}} e^{-\frac{(x-x_c)^2}{w^2}}
\]

where \(y_0\) is the offset of the spectrum, \(x_c\) is the center of the peak of the Gaussian curve, \(w\)
is the width the Gaussian curve and \(A\) is the area under Gaussian curve.

After the Gaussian fit is done, the software will automatically calculate the height, the area
under the Gaussian fit, the width, and the center of the peak of the fit of each Gaussian fit.
These heights were used to calculate the ratio of the heights of the two peaks. The Gaussian
spectral function was used because it provides an acceptable matching fit of spectral data with
good standard errors for the peak amplitude, peak center, and full width at half maximum [1].

3.1.3 Time evolution of the spectrum

A continuous source, He-Ne or argon ion laser, was used to irradiate a single spot on the leaf.
A problem that one may encounter here is the evolution of the spectrum in terms of time
Figure 3-2: Gaussian fit of the chlorophyll fluorescence spectrum used for the analysis of the spectrum.
response. To overcome this problem, an investigation of temporal variations of the chlorophyll fluorescence was needed to observe the variations in the fluorescence spectrum as a function of time, using a continuous excitation source. Spectra were recorded at fixed time intervals (of typically 2 seconds) during irradiation, using the Ocean Optics software. Figure 3-3 shows three spectra recorded after 4, 12, and 24 seconds. It was noticed that the intensity of the red and far red peaks of the chlorophyll fluorescence spectrum decreased with time. To make a detailed study of temporal evolution of the fluorescence spectrum, a spectrum was recorded every two seconds. While recording the spectra of the chlorophyll fluorescence, it was observed that there was a fast increase of the fluorescence intensity. The maximum fluorescence intensity was reached within approximately two seconds as noticed in the previous section, after which the intensity decreased more slowly.

For the analysis of time-resolved spectra, the values of the wavelengths of the peaks were detected. The wavelength value of each peak was then associated with a specific channel of the Ocean Optics spectrometer, recording the intensity of the specific wavelength. Every two seconds the data of the variation of the peaks and the full spectrum of the chlorophyll fluorescence are collected automatically. Those data were then imported into the Microcal Origin software. A second order exponential decay fit was done for each peak recorded, as shown in Figure 3-4. These decay fits are expressed by the equation:

$$ Y = Y_0 + A_1 e^{-\frac{(t-t_0)}{\tau_1}} + A_2 e^{-\frac{(t-t_0)}{\tau_2}} $$

(3.2)

where $Y_0$ is the offset, $t_0$ is the center of the peak, $A_1$ and $A_2$ are the amplitudes and, finally, $\tau_1$ and $\tau_2$ are the time decay constants. In order to obtain the perfect fit of the decrease of chlorophyll fluorescence, the values of $Y_0$ and $t_0$ were kept constant at a value of zero. The solid curve in Figure 3-4 shows how the exponential second order fit of the far red peaks of the chlorophyll fluorescence decay was done.
Figure 3-3: Variation of the chlorophyll fluorescence intensity spectra with time. The three spectra were recorded at fixed different time: after 4 seconds, after 12 seconds and after 24 seconds.
Figure 3-4: Graph of the chlorophyll fluorescence decrease of the red peak. Experimental points are for the decrease of the red peak (dots) and numerical analysis for the exponential decay fit of the peak (solid line).
3.2 Fluorescence Imaging

3.2.1 Setup for continuous wave and pulse laser measurements

Two fluorescence imaging systems were used for acquiring the images and detecting the chlorophyll fluorescence of fruit and leaves, namely apples (Golden Delicious) and *Nicotiana tabacum* (tobacco) leaves. The apples were acquired from the supermarket. The *Nicotiana tabacum* plants were grown in a glass house by the Institute of Wine Biotechnology, at the University of Stellenbosch.

The fluorescence imaging setup using He-Ne laser is shown in Figure 3-5. The first system used a continuous source of excitation. The CW excitation source used was a He-Ne laser of 5 mW output power operating at a wavelength of 632.8 nm. As the beam size of the He-Ne laser is very small, a bi-concave lens of −25 cm focal length was placed in front of the laser beam to expand it over a large area of the sample. An intensified CCD camera (Xybion electronic systems model ISG-250-CGRX-3) was used to image the chlorophyll fluorescence from the sample being illuminated. The camera was set at an angle of about 40 degrees with respect to the laser excitation beam. A long pass filter (RG 645) was used to cut off the scattered laser light, and leaving only the passage of the fluorescence signal of the sample to be recorded.

To capture an image, the camera and the image card in the computer had to be triggered. The camera can be triggered externally by providing a negative edge TTL signal of typical 50 ns duration. The camera takes approximately 60 ns to recover before it can be re-triggered, and the gate duration period selected must be less than the period between successive triggers (otherwise the camera switches automatically to a non-gate mode). In addition, the camera has an internal delay that can be set. By adjusting the delay, the external trigger timing can be modified. The period between external trigger pulses must therefore be greater than the delay.

The camera gate and the card were triggered using a four channel digital delay/pulse generator from Stanford Research (model DG535), which supplied a square TTL signal of 4 V to the camera and to the card inside the computer. The images were stored on the computer. For analyses of the recorded image, a program was used to transform the image into a text file and then plot it with the Microcal Origin software.

The second setup used for the detection of the chlorophyll fluorescence imaging was more
Figure 3-5: Experimental setup of chlorophyll fluorescence imaging using a He-Ne laser as continuous excitation source.
complex than the previous one. Figure 3-6 shows the layout of the laser induced fluorescence imaging setup using an excimer laser. The source of irradiation used was the XeCl excimer laser, operating at a wavelength of 308 nm and with a pulse duration of about 25 ns. At the time that this experiment was carried out the power output of the excimer laser was unstable, with the output fluctuating between 45 mJ and 63 mJ. A divergent lens and screen with a hole (diaphragm) were inserted between the source of excitation (excimer laser) and samples (leaves) in order to control the size of the beam. By moving the lens and the screen it was possible to increase or decrease the beam size of the laser on the leaves. The capturing of the image was done in reflection. An intensified CCD camera (Xybion electronic systems model ISG-250-CGRX-3) was used to image the fluorescence of the sample. A UV cut off filter made from perspex material was inserted between the leaves and the camera in order to cut off light from the laser source and allow the visible fluorescence to pass. Perspex is well suited to block the UV light at 308 nm and pass the visible light, and can therefore be used to separate the excimer wavelength and the chlorophyll fluorescence. A set of four narrow bandwidth interference filters were used: the wavelengths were 450 nm, 520 nm, 690 nm, and 730 nm, and each filter had a bandwidth of 5 nm. These filter wavelengths correspond to the peaks of plant material fluorescence. The filters were used individually to record images at the different fluorescence peaks. The filter was inserted inside the camera by first removing the camera lens and iris, fixing the filter internally and then replacing the lens.

In order to synchronise image capture, the delay/pulse generator was used to trigger the camera and A/D card inside the computer in the same way as described above. The delay generator also served as a trigger for the laser. The delay/pulse generator supplying a maximum square TTL signal of 4V could not trigger the laser directly because of the low voltage, thus an electronic circuit was used inside the laser to enable the delay generator to trigger the excimer laser. The desired image was recorded by first triggering the laser and delaying the trigger of the camera and card, and simultaneously controlling the camera intensifier. In addition, the external trigger timing was adjusted by the camera delay, to allow the camera to wait for an event to occur before capture of an image. The camera begins image capture at the falling edge of the trigger pulse. Range and duration controls of the camera permit the duration of the camera integration period to be set. As stated above, the external trigger pulse repetition
must be such that the selected delay and the gate duration period are less than the period between successive triggers, in order to avoid a non-gated mode for the camera when capturing an image. The gate duration was set between 25 ns and 100 ns and the laser repetition rate at 1 Hz.

3.2.2 Image processing

The images acquired by the CCD camera were transferred to the computer. To facilitate the interpretation of the captured images, an imaging processing program was written in Matlab (version 6.5). The program takes the images saved as a bitmap image file, as shown in Figure 3-7a, and then transforms the image file to a text file. These text files can be presented in Microcal Origin in the form of a matrix. So the text file was therefore imported into the Origin
software and the image plotted as contour-colour fill, as indicated in Figure 3-7b.

For further analysis of the image, a second program for image processing was written. This program served to clean the images. The program takes the original bitmap image and removes isolated pixels from the image. The program will then save the data remaining from the original image. These data were saved not only as a bitmap image but also as a text file. Depending on the clarity of the image, one may indicate how many steps of cleaning are required. The text files of the cleaned picture can then be plotted as a colour contour image. Figures 3-7b and 3-7c show examples of images of chlorophyll fluorescence of a tobacco flower before and after the image had been through the image cleaning process.

A third image processing program was written for the evaluation of ratios between the two images. The program was written in Matlab software (version 6.5). To obtain the ratio of two images, the text files of the two images obtained in the first image processing program are imported into Matlab software. The program converts these files to matrices and uses the operation of matrix division to divide the elements of one matrix by the corresponding element of the second matrix. The resulting matrix is saved in the form of a text file. The text file is then imported into Microcal Origin to plot the result in as a colour contour image.
Figure 3-7: a) Image of a Nicotiana tabacum flower irradiated by the He-Ne (632.8 nm and output power of 5 mW). b) Image of a Nicotiana tabacum flower irradiated by a continuous source of excitation, He-Ne laser, plotted in colour contour. c) Image of a Nicotiana tabacum flower irradiated by He-Ne laser, plotted after it went through the first cleaning step of cleaning procedure. d) Image of a Nicotiana tabacum flower irradiated by the He-Ne laser, plotted after it has gone through the second cleaning step of the cleaning procedure.
Chapter 4

Experimental Results and Discussion

4.1 Chlorophyll Fluorescence Spectra

In order to study the spectrum of chlorophyll fluorescence emission of tobacco leaves the method of single spot fluorescence measurement was used, as discussed in Sections 3.1.1 and 3.1.2. The detection of the fluorescence was done in transmission mode as described in the experimental setup of fluorescence spectroscopy (Section 3.1.1). The results plotted in Figure 4-1 show the chlorophyll fluorescence spectrum of a tobacco leaf irradiated by the argon ion laser operating at 455 nm. In contrast, Figure 4-2 shows the spectrum of a tobacco leaf obtained using the He-Ne laser at 632.8 nm as the source of excitation. Although irradiated by different laser sources, the chlorophyll fluorescence spectra of the tobacco leaves exhibit the same shape. For each source of excitation, the spectra exhibit the typical double peak curve, with maxima at about 690 nm and 735 nm. In addition, the relative intensity of fluorescence of the red band is stronger than that of the far red band. This shape of the spectrum corresponds to the shape of chlorophyll-a fluorescence when isolated.

The 645 nm long pass filter that was used was not able to cut off the He-Ne laser light completely. About 10% to 15% of the beam was transmitted through the filter. As a result, a narrow peak around 632.8 nm was observed, as shown in Figure 4-2.

In contrast to the chlorophyll fluorescence spectra displayed in Figures 4-1 and 4-2, Figure 4-3 gives another shape of the chlorophyll fluorescence spectrum. The spectrum in Figure 4-3 was obtained by irradiating Ligustrum Japonicum leaves with the He-Ne laser at 632.8 nm.
Figure 4-1: Chlorophyll fluorescence spectrum of Nicotiana tabacum leaves excited by the argon ion laser at 455 nm. The 550 nm long pass filter was used to cut off the laser light.
Figure 4-2: Chlorophyll fluorescence spectrum of Nicotiana tabacum leaves excited by the He-Ne laser at 632.8 nm. The long pass filter of 645 nm was used to cut off the laser light intensity (10% to 15% of the laser intensity is transmitted).
The recorded fluorescence spectrum has a totally different shape to that of the fluorescence spectrum of the tobacco leaves obtained previously. The two fluorescence bands still appear but the relative intensity of the far red band, F735, was now stronger than that of the red band, F690.

The well known process of fluorescence re-absorption may be one explanation for the differences between the shape of the chlorophyll fluorescence of the *Nicotiana tabacum* leaves and the *Ligustrum japonicum* leaves. The changes in the shape of the chlorophyll fluorescence emission spectra at room temperature are due to the re-absorption process that affects the red band of chlorophyll fluorescence. In vivo, the chlorophyll fluorescence emission spectrum of the red band strongly overlaps with the maximum of the leaf absorption spectrum, as indicated previously in Figure 2-5 [12, 14]. As a result, a significant and large part of the red band is re-absorbed by chlorophyll molecules inside the leaf before it can be detected outside the leaf.

A second reason relating to the change in the chlorophyll fluorescence spectrum may involve the shape, the anatomy or the physiological state of the leaf. The *Ligustrum japonicum* leaves are thicker than the tobacco leaves. They are also dark green leaves while tobacco leaves are more light-green. An increased thickness of the leaf will increase the re-absorption of the red band. As a result one may end up with a drastic decrease in the relative intensity of the red chlorophyll fluorescence in *Ligustrum japonicum* leaves compared to that of tobacco leaves. These observations regarding the difference in shape of the chlorophyll fluorescence spectra between the tobacco leaves and *Ligustrum japonicum* leaves can also be justified by the difference in concentration of chlorophyll inside these leaves. The *Ligustrum japonicum* leaves were bright green, which is a sign of a high chlorophyll concentration, compared to the tobacco leaves which were light green [1, 12]. Higher chlorophyll concentration also causes more re-absorption. A third reason for the difference in spectral shape may arise from the fact that the *Ligustrum japonicum* leaf that was used was a leaf which had been picked from the plant, whereas the tobacco leaf was measured, in situ, on the potted plant. This *Ligustrum japonicum* leaf could therefore have undergone water stress, which may contribute to the changes in intensity of the red chlorophyll fluorescence.

In order to achieve good analysis of spectra and to obtain better information from the spectrum the deconvolution option was used, by means of a Gaussian fitting method [1]. By
Figure 4-3: Chlorophyll fluorescence spectrum of Ligustrum japonicum leaves that are thicker than tobacco leaves. The He-Ne laser at 632.8 nm was used as source of excitation. A long-pass filter of 645 nm was used to cut off the laser wavelength.
applying the multi-peak Gaussian fit procedure from Microcal Origin to the spectrum, specifying
the number of peaks that need to be fitted, and also indicating their positions, the result of the
double Gaussian fit shown in the Figure 3-2 previously, are obtained directly.

Those double fits provide ample information for the spectrum, for instance the curve-fitting
parameters such as peak center, peak height, bandwidth (FWHM) and the area under each
Gaussian curve [1], [14]. Combining the two Gaussian curves, the software produces the re-
sultant curve (red curve in Figure 3-2), which is an almost the perfect fit to the measured
spectrum. For this work all analysis of spectra was done using Gaussian fits and the width,
height and the peak center of the peaks were recorded.

The single spot fluorescence measurement of intact leaves may provide only information
about a specific spot on the leaf. In order to have an idea about spatial distribution of the
fluorescence and to check if the information provided by single spot measurements reflects
the behaviour or the status of the total leaf, a point data measurement at different spots of
the leaf was done using the He-Ne laser. Spectra from different spots were recorded and the
deconvolution of each spectrum was then made by means of the Gaussian peak fit, providing
values of the height of the Gaussian fit curve of the red and far red band, as well as the spectral
full width at half maximum. Figure 4-4 shows the plot of the widths of the red and the far red
fluorescence bands for different spots on a leaf. The data plotted in Figure 4-4 were obtained
from spectra recorded on the Ligustrum japonicum leaves. The widths of the red and of the
far red fluorescence bands seem to be constant. For the red band, the value of the widths for
different spots range between 26 nm and 27 nm, while for the far red band the widths range
between 42 nm and 43 nm.

One of the most important parameters in the analysis of the chlorophyll fluorescence spec-
trum is the ratio of the intensities of the red and the far red fluorescence, F690/F735. Measure-
ments of the height of the Gaussian peak fit of the red band and that of the far red band allow
us to calculate this fluorescence ratio. The ratio varies from point to point for measurements
taken on the same leaf. Figure 4-5 shows that these ratios vary between 0.59 and 0.7 for the
Ligustrum japonicum leaf. This shows that the ratio may change from one spot to the other
for the same leaf, these variations of the ratio are about 15%, as shown in Figure 4-5. This
does, however, indicate that point data measurements of chlorophyll fluorescence do not provide
Figure 4-4: Graph of the full width at half maximum (FWHM) of the fluorescence bands for the different spots of Ligustrum japonicum leaf for the red (open circle) and far red (solid squares) chlorophyll fluorescence on one leaf.
Figure 4-5: Variation of F690/F735 ratio of chlorophyll fluorescence at different spots on one Ligustrum japonicum leaf.

exactly identical measurement at different spots. In addition, the ratio F690/F735 depends on the chlorophyll content [1]. It decreases with the increase of chlorophyll content, therefore the variation of these fluorescence intensity ratios noticed may also be the result of slight differences in the chlorophyll content distribution in the intact leaf, because of the anatomy of the leaf.

4.2 Fluorescence Quenching

When using the He-Ne laser as a constant excitation source to irradiate the leaves it was observed that during data acquisition of the spectrum the chlorophyll fluorescence spectrum
changed continuously. The chlorophyll fluorescence reached a maximum within approximately two seconds, followed by a continuous decrease of the intensity of both the red and the far red bands. The spectra of the chlorophyll fluorescence presented in Figure 4-6 show the variation of the red and far red bands for a *Ligustrum japonicum* leaf. Spectra were recorded every two seconds. This variation of the chlorophyll fluorescence appears to be related to changes in the photosynthetic activity of the leaf [12]. The intensity of the red and far red chlorophyll fluorescence is inversely proportional to the photosynthetic activity. A decrease in chlorophyll fluorescence indicates therefore an increase of the photosynthetic activity of a leaf.

When a dark-adapted leaf is suddenly illuminated by a constant source of irradiation, the PS II reaction centres are progressively closed. This will lead to an increase of the chlorophyll fluorescence. However, after a time of about 2 seconds, the chlorophyll fluorescence decreases for a few minutes before it reaches the steady state. This phenomenon is known as fluorescence quenching. This decrease of the chlorophyll fluorescence is due to the fact that the laser source will activate the enzymes involved in the carbon metabolism and also activate the opening of small apertures in the epidermis of the leaf (stomata) [3]. In such way the rate at which the electrons are used by the photosystem II will increase and will enhance photosynthetic activity. The efficiency with which energy is converted to heat will increase as well. The increased rate in both of these pathways results in a decrease in the fluorescence intensity.

### 4.2.1 Results of fitting: non Gaussian and Gaussian

For each spectrum recorded, the values of the two chlorophyll fluorescence peaks as measured are registered. After collecting those peak values, a plot of variation of each peak as a function of time was made. The black squares shown in Figure 4-7 represent the decrease of the far red peak of the chlorophyll fluorescence while the green circles indicate the decrease of the red peak. This decrease of the chlorophyll fluorescence bands can be explained by two processes. The first is a fast decrease of the chlorophyll fluorescence. It is related to the primary photochemistry of the photosystem II. The second process is a slow decrease of chlorophyll fluorescence before it reaches the steady state. These slow kinetics are related to interaction between processes in thylakoid and in the reductive carbon cycle of the stroma.

An exponential decay fit of second order was done on the decrease of intensity over time
Figure 4-6: Time resolved spectra of chlorophyll fluorescence. Spectra were recorded from the *Ligustrum japonicum* leaf irradiated by a He-Ne laser of 633 nm wavelength and 5 mW output power. Spectra were recorded every two seconds.
Figure 4-7: Graph of quenching of chlorophyll fluorescence for the red and far red peak intensities as measured fitted by a second order exponential function. The He-Ne laser of 5 mW power output was used as source of irradiation of the Ligustrum japonicum leaf.
for each peak decrease using the Microcal Origin software (see Figure 4-7). The fit function is
given by Equation 3.2. Although the exponential decay fit may also be done in third order, in
this case the second order gives the best fit that can be obtained. The exponential decay fit
allows us to extract the parameter $\tau_1$, which is related to the fast process of the chlorophyll
fluorescence quenching, and parameter $\tau_2$, which is related to the slow process.

Furthermore, a double Gaussian fit was done on each recorded spectrum and the height of
each Gaussian peak was saved. The height variation of the Gaussian peaks was plotted as a
function of time, as shown in Figure 4-8, and the values of $\tau_1$ and $\tau_2$ were extracted using the
exponential decay fit method. The same phenomena are observed as in the case of the originally
measured chlorophyll peaks: a fast and a slow quenching process are noticed.

To get an accurate fit, the values of $Y_0$ and $t_0$ were fixed to zero. The values of all factors
found for the exponential decay fit are summarised in Table 4.1. The study of these exponential
fits showed that the values of $\tau_1$ were much smaller than those for $\tau_2$. However, the values of
$\tau_1$ for the red peak at 690 nm were larger than those for the far red peak at 735 nm, whereas
the values of $\tau_2$ the for red peak were smaller than those for the far red peaks. These differences
were noticed not only on the exponential decay fits for the chlorophyll fluorescence peaks but
also on the fits for the Gaussian peaks. Moreover, as expected, there is a slight difference
between the exponential decay fit of chlorophyll fluorescence peaks and Gaussian peaks, in
term of $\tau_1$ and $\tau_2$ values.

<table>
<thead>
<tr>
<th></th>
<th>peak 690 nm</th>
<th>peak 735 nm</th>
<th>Gaussian peaks 690 nm</th>
<th>Gaussian peaks 735 nm</th>
</tr>
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<tbody>
<tr>
<td>$Y_0$</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$t_0$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$A_1$</td>
<td>3717.95</td>
<td>4422.37</td>
<td>3341.52</td>
<td>4416.14</td>
</tr>
<tr>
<td>$\tau_1$</td>
<td>16.49</td>
<td>15.57</td>
<td>17.66</td>
<td>16.19</td>
</tr>
<tr>
<td>$A_2$</td>
<td>1089.69</td>
<td>1516.02</td>
<td>754.85</td>
<td>1507.59</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>491.27</td>
<td>569.27</td>
<td>487.82</td>
<td>629.70</td>
</tr>
<tr>
<td>Ski factor</td>
<td>1232.37</td>
<td>1264.45</td>
<td>1109.82</td>
<td>794.23</td>
</tr>
</tbody>
</table>

Table 4.1: Summary of the values of parameters used in the second order
exponential decay fit (see Equation3.2, Section 3.1.3)
Figure 4-8: Graph of the quenching of chlorophyll fluorescence for Gaussian fit peaks of the red and far red peaks fitted by a second order exponential function. The He-Ne laser of 5 mW power output was used as source of irradiation of the Ligustrum japonicum leaf.
4.2.2 Intensity dependence of the time constant

An investigation into the variation of chlorophyll fluorescence with the change of laser output power was carried out. A He-Ne laser of 5 mW power output was used to irradiate tobacco leaves. The output power was changed by placing neutral density filters in front of the output of the He-Ne laser. Two different neutral density filters were used: a ND0.1 filter, transmitting 80% of the intensity of the He-Ne laser, which is about 4 mW, and a ND0.5 filter, transmitting 32% which is about 1.6 mW. In Figure 4-9 the black line represents the chlorophyll fluorescence quenching of the tobacco leaf using 5 mW irradiated intensity, the green line when using 4 mW and the blue line when using 1.6 mW. The data plotted in Figure 4-9 were recorded for the red chlorophyll fluorescence peak of tobacco leaves. In this work it was noticed that the intensity of the chlorophyll fluorescence was affected by the output power of the laser. The fluorescence intensity varied proportionally to the output power of the laser. As the output power of the He-Ne laser is reduced, the intensity of the chlorophyll fluorescence of the red and far red bands decreases. But the shape of the fluorescence quenching remains the same, with the fast and the slow quenching remaining distinct. In addition, the chlorophyll fluorescence quenching of tobacco leaves excited by the He-Ne laser at 5 mW output power remains above that of the leaves excited with 4 mW and 1.6 mW and, similarly, the fluorescence quenching at 4 mW was above that at 1.6 mW (Figure 4-9).

\[
\begin{array}{|c|c|c|}
\hline
 & 5 \text{ mW} & 4 \text{ mW} & 1.6 \text{ mW} \\
\hline
\tau_1 & 13.91 & 15.51 & 18.65 \\
\hline
\tau_2 & 197.74 & 184.81 & 429.55 \\
\hline
\end{array}
\]

Table 4-2: Variation of chlorophyll fluorescence parameters \( \tau_1 \) and \( \tau_2 \) with increase of the output power of He-Ne.

All data of fluorescence quenching reported were recorded by using the single point data measurement method and the fluorescence quenching curves shown in Figure 4-9 were recorded at different spots of the tobacco leaf (e.g. each fluorescence quenching curve was recorded at a
different single spot using a specific power). When a leaf is irradiated with 5 mW power, more chlorophyll molecules are excited compared to 4 mW and 1.6 mW power. This will lead to a higher chlorophyll fluorescence for higher incident radiation power. This is in accordance with Equation 2.2, which shows that an increase in the incident radiation power produces an increase in fluorescence intensity. However, it was found that the values of the $\tau_1$ and $\tau_2$ increase with decrease of the output power of the laser, as indicate Table 4-2.

The study of chlorophyll fluorescence quenching with variation of the power output of the He-Ne laser was also carried out for one single spot on the leaf. A spot on the leaf was irradiated by the He-Ne laser with a power of 4 mW for a duration of 3 minutes. During this time spectra of the chlorophyll fluorescence quenching were recorded. After recording the spectra, the laser is switched off for a minimum time of 5 minutes to give the leaf time to recover. The experiment was then repeated in the same way, by changing the power output of the laser to 1.6 mW and then finally to 5 mW, according to the same method (still irradiating the leaf at the same spot). The chlorophyll fluorescence quenching curves were compared. It was found that they were overlapping. The results were totally different from those obtained in Figure 4-9. For instance, the chlorophyll fluorescence quenching curve using the laser output power of 4 mW was higher than the one using a laser output power of 5 mW. These results contradicted those obtained above.

In order to try to find an explanation for these differences, a tobacco leaf was irradiated with a laser power of 4 mW. The time of the laser on an individual leaf spot was set at 10 seconds while the recovery period was set at 5 minutes. Results shown in Figure 4-10 indicate that the intensity of the fluorescence does not remain the same when the leaves were again irradiated at the same spot. The chlorophyll fluorescence intensity, under these conditions, diminishes each time the leaf is re-irradiated on the same spot. This indicates that the leaf does not recover totally within 5 minutes and when irradiated a second time at the same spot the intensity of the fluorescence will be lower than previously. These differences in intensity result from the quenching of the chlorophyll fluorescence. Additionally, under constant irradiation of the leaf for a longer period, the absorbed energy may bleach certain chlorophyll molecules. Therefore the number of chlorophyll molecules which will fluoresce diminishes, and fluorescence intensity will decrease.
Figure 4-9: Chlorophyll fluorescence quenching of Nicotiana tabacum leaf for various power level of the He-Ne laser for the red peak at 690 nm: 5 mW (black curve), 4 mW (olive curve) and 1.6 mW (blue curve). The red curve indicate the exponential decay fit of second order.
Figure 4-10: Graph of intensity variation of chlorophyll fluorescence when a tobacco leaf is irradiated with a He-Ne laser at the same spot. The laser was kept at a single spot for 10 seconds. The leaves were then left for 5 minutes to recover, and then re-irradiated.
4.2.3 Quenching with shorter wavelength excitation

Chlorophyll fluorescence quenching of tobacco leaves was also investigated by illuminating tobacco leaves with wavelengths shorter than that of the He-Ne laser. Quenching measurements were carried out by irradiating leaves with a CW, line tunable argon ion laser, operating at 455 nm and with an output power 5 mW. The purpose of this investigation was to detect the impact of the shorter wavelength excitation on chlorophyll fluorescence quenching. The results showed the same characteristics as previously. The chlorophyll fluorescence declines by a fast decrease process followed by a slow decrease process before the fluorescence reaches the steady state. An exponential decay fit of second order was done on the chlorophyll fluorescence decline, as indicate the Figure 4-11, in order to determine the time decay parameters $\tau_1$ and $\tau_2$.

The results of the fit show that the fast process of fluorescence decline is more rapid in the case of the argon ion laser used as excitation source than in the case of the He-Ne laser. The values of the parameter $\tau_1$ are 8.93 s and 9.01 s for the red and far red peaks respectively. In the case of the He-Ne laser the values of $\tau_1$ are 13.90 s and 15.09 s for the red and far red peaks respectively, as show in the Table 4-3.

However, the slow process of fluorescence decline is slower in the case of the argon ion laser than in the case of the He-Ne laser. The values of parameter $\tau_2$ are 750.2 s and 1177 s for the argon ion laser and 184.8 s and 263.4 s for the He-Ne laser as source of excitation. Table 4-3 give a summary of determined values for parameters $\tau_1$ and $\tau_2$.

<table>
<thead>
<tr>
<th>a) Argon ion laser</th>
<th>b) He-Ne laser</th>
</tr>
</thead>
<tbody>
<tr>
<td>peak 690 nm</td>
<td>peak 735 nm</td>
</tr>
<tr>
<td>$\tau_1(s)$</td>
<td>8.93</td>
</tr>
<tr>
<td>$\tau_2(s)$</td>
<td>750.2</td>
</tr>
</tbody>
</table>

Table 4-3: Values of $\tau_1$ and $\tau_2$ of chlorophyll fluorescence for red and far red peaks obtained when using: a) Argon ion laser, b) He-Ne laser

The chlorophyll fluorescence quenching of tobacco leaves indicates the presence of two processes: a fast and a slow chlorophyll fluorescence decrease. The chlorophyll fluorescence quenching exhibited these two processes irrespective of whether the longer or the shorter wavelength...
Figure 4-11: Graph of quenching of chlorophyll fluorescence for the red and far red peaks fit by a second order exponential decay function. The argon ion laser of 5 mW power output was used as source of irradiation of a Nicotiana tabacum leaf.
excitation was used. However, the fast process appears to be faster in the case of the argon ion laser than in the case of the He-Ne laser. For the slow process the fluorescence takes longer to reach the steady state when the Argon ion laser is used.

4.3 Pulsed Laser Fluorescence Imaging

The setup used for recording images of the chlorophyll fluorescence using a pulsed laser was shown in Figure 3-6. For this experiment, the perspex filter and the filters set in front of the camera were removed. An apple was exposed to the excimer laser beam at ambient light. It was difficult to perceive the fluorescence from the image recorded. The presence of the background light caused the visibility of the captured image to be poor as illustrated by Figure 4-12a. The apple was then dark-adapted and the image capture was performed in total darkness in order to exclude interfering background radiation such as sunlight and laboratory lights. One of the aims of spectroscopy is to discriminate the excitation light from the emitted light. Therefore a delay time between the trigger of the laser and the trigger of the camera was set at 10 µs to avoid the capture of scattered laser light as the camera is not wavelength selective. The image shown in Figure 4-12b indicates a fluorescence that was not detectable under ambient light.

This laser induced fluorescence imaging setup with the excimer laser was used to detect the fluorescence of plant material and to investigate the lifetime of the fluorescence. An apple was irradiated with the excimer laser at 308 nm wavelength and a pulse duration of 25 ns. As indicated previously (in Section 3.2.1) the gate of the camera was set 20 % of range 1. This gave a time window of approximately 40 ns. A delay generator (Stanford and Research, model DG535) was used to set the relative delay between the laser trigger and the camera. A sequence of images was recorded at different delays, as indicated in Figure 4-13. A reflecting mirror for 308 nm was inserted in front of the camera to reduce the intensity of scattered light. At a delay of 367 ns a bright scattering of the laser on the image can be seen (top left corner of the image), therefore the laser pulse and the gate of the camera are overlapping or coincide at that moment. This is due to the angular dependency of the scattering, which is brightest for angles close to normal incidence. At 367 ns delay, where the laser and camera gate overlap, a delay of 10 ns was added, to increase the delay at 377 ns, and an image was recorded. Therefore at a
Figure 4-12: a) Image of apple in ambient light. b) Fluorescence imaging of apple illuminated by the excimer laser at 308 nm and recorded 10 µs after the laser was fired.
delay of 377 ns the image is captured approximately 10 ns after the laser pulse. This procedure was repeated. Figure 4-13 summarises the images recorded for increasing camera delay.

The scattered intensity decreases strongly from images Figure 4-13a to 4-13b while the fluorescence emitted by the apple becomes more dominant. Two distinct bright spots appeared when the delay was increased. Figure 4-13 indicates that after 30 ns delay (c) the fluorescence emitted by the apple became visible, and became increasingly clear as the delay increased. Further increase in the delay beyond 447 ns, to capture the image more than 80 ns after the laser pulse, totally suppressed the fluorescence. The image in Figure 4-13h shows the decrease of the fluorescence captured at 70 ns after the laser pulse towards large decay. The distinct spots showing up in fluorescence were not visible in the scattered light or by visual inspection. The brightness of each image was adjusted by the intensifier and camera gain to avoid saturation of the image. The absolute brightness of different images can therefore not be compared. In reality, the brightness of the images decreased strongly from (a) to (h). The relative delay of the camera gate in relation to the laser pulse seems to indicate that the lifetime of the fluorescence is of the order of 80 ns. Normally the chlorophyll fluorescence lifetime is less than that value [20]. This difference in the observed lifetime arose from the adjustment of the camera intensifier and gain. Although the results obtained for the fluorescence lifetime of apples were not conclusive, they showed that the method could be used to investigate the lifetimes of other plant materials.

These observations have demonstrated that, on the one hand the method of delaying the camera gate in respect to the laser pulse can be used to discriminate the laser scattering from the fluorescence of the plant material and, on the other hand, it allows the determination of the fluorescence lifetime.

4.4 Investigation of GFP Expression in Plant Tissue

The effect of GFP on chlorophyll fluorescence was investigated by laser induced chlorophyll fluorescence of tobacco leaves.

Two tobacco plants were grown in separate pots in the laboratory. These plants were kept at room temperature and in a place where they were exposed to sunlight. After two weeks of growth the leaves were mature and a GFP gene was introduced into some of the leaves of one
Figure 4-13: Sequence of images exhibiting the fluorescence of the apple. Images were taken with delays of: a) 367 ns, b) 377 ns, c) 387 ns, d) 397 ns, e) 407 ns, f) 417 ns, g) 427 ns, h) 437 ns.
of the plants. Two days after the GFP was introduced experiments were carried out on the plants. The experiments had to be done within 7 days since after that period the expression of the GFP in leaves vanishes and fluorescence of the GFP may not be detected or visualized.

The investigation of GFP expression in tobacco leaves was done first by using the chlorophyll fluorescence imaging setup to acquire images. The excimer laser at 308 nm and 25 ns pulse duration was used for this experiment. Second, the chlorophyll fluorescence spectroscopy setup was employed to record the fluorescence spectrum of the leaves using the He-Ne and the argon ion lasers.

In addition, the leaves irradiated by the UV light of the excimer laser were used in the chlorophyll fluorescence spectroscopy experiment in order to look at the influence of UV light on the fluorescence of tobacco leaves with and without GFP treatment.

4.4.1 Spectral results

Chlorophyll fluorescence emission spectra of dark-adapted tobacco leaves, generated by the laser induced chlorophyll fluorescence spectroscopy system described in Figure 3-1, produced the typical double peaked spectra with maxima at about 690 nm and 735 nm. Figure 4-14a presents spectra of two tobacco leaves irradiated by the He-Ne laser. The orange curve represents the spectrum of a tobacco leaf in which GFP gene was inserted while the black curve is the spectrum of the control leaf. The shape of the chlorophyll fluorescence of those two spectra shows that the intensity of the measurable chlorophyll fluorescence in the red band is much higher than that of the far red band. In addition, the intensity of the chlorophyll fluorescence of the control tobacco leaf is higher than that of the leaf treated with GFP. The spectra in Figure 4-14b present a different situation. Here the chlorophyll fluorescence intensity of the tobacco leaf treated with GFP is higher than that of the control tobacco leaf. This incompatibility of results was due to the fact that the fluorescence spectra were only measured on a single leaf spot, and a single leaf spot provides only specific fluorescence information on that particular leaf spot, which may not be a true reflection of the fluorescence intensity on the whole leaf. In addition, the detection system used (see Figure 3-1) was not totally reproducible. The intensity of chlorophyll fluorescence did depend on the closeness of the leaf to the fluorescence filter. The geometry or the shape of the leaf therefore influences the intensity
of the fluorescence. The leaves were not totally flat, they were somewhat bumpy, resulting in varying fluorescence intensities for different spots on the leaf. These differences were noticed on the leaves irradiated by the UV excimer laser and as well on the non-irradiated leaves. The comparison of the total intensity of the chlorophyll fluorescence spectra was therefore not a good way to investigate the GFP expression in tobacco leaves.

Determination of the ratios of the red to far red fluorescence appeared to be a better way to study the GFP influence on chlorophyll fluorescence. As before, a Gaussian fit was made for all the recorded fluorescence spectra and the ratios were then calculated from these fits. Table 4-4 summarises the values of the ratios of the red to the far red bands, F690/F735.

As mentioned previously, images of tobacco leaves were taken before the spectra were recorded. Some brighter spots did show up on the leaves, and spectra of those bright spots, called “hot spots”, and spectra of the normal spots, called “cold spots”, were recorded and analysed.

In the case of the tobacco leaves which were exposed before to the excimer laser light, the ratio F690/F735 of the control leaves was higher than that of leaves with GFP. Table 4-4 shows that the F690/F735 ratio on different leaves from the control tobacco plants (Table 4-4b) and at different spots of a leaf (Table 4-4c) is higher than in the case of the tobacco leaves with GFP genes. A decrease of about 15% to 25% in the ratio of red to far red fluorescence was noticed when GFP genes are expressed in the tobacco leaves.

In contrast for non previously irradiated leaves (Table 4-4a), the F690/F735 ratio appears to be the opposite to what was mentioned above. The value of the ratio of red and far red chlorophyll fluorescence of the tobacco leaves with the GFP gene inserted is about a factor of 10% higher than the value of the ratio for the tobacco control leaves.
Figure 4-14: a) Laser induced chlorophyll fluorescence spectra of tobacco leaf treated with GFP (orange spectrum) and control leaf (black spectrum). b) Laser induced chlorophyll fluorescence spectra of tobacco leaf treated with GFP (violet spectrum) and control leaf (red spectrum).
Table 4-4: Results of the chlorophyll fluorescence intensity ratio of red and far red band taken on:
a) non-irradiated leaves, b) irradiated leaves, c) spot of irradiated leaves, and d) averages of ratios

<table>
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<th>Spots</th>
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<td>hot 1</td>
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<td>1.87</td>
</tr>
<tr>
<td>hot 2</td>
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<tr>
<td>cold 1</td>
<td>1.63</td>
<td>1.98</td>
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<tr>
<td>cold 2</td>
<td>1.73</td>
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<tr>
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</tr>
<tr>
<td>Average</td>
<td>1.65</td>
<td>1.99</td>
</tr>
</tbody>
</table>

The ratios indicate that in the case of the leaves treated with GFP gene, and not irradiated with UV, the GFP may be interfering with the photosynthetic process. The F690/F735 ratio of chlorophyll fluorescence of non-irradiated tobacco leaves is equal to an average value of 1.55 for the control leaves, while this average is about 1.65 for leaves with GFP. In the present study, tobacco leaves treated with GFP showed an increase of about 6% in chlorophyll fluorescence F690/F735 ratio compared to the control leaves. This means that the magnitude of the 690 nm chlorophyll fluorescence band has increased. An increase in peak height can be correlated with a decrease in photochemical electron transport involved in both reaction centres PS I and PS II, and therefore decrease in the photosynthesis system activities. In addition, since the chlorophyll fluorescence is inversely proportional to the photosynthetic activity [21], it can be concluded that the presence of the GFP gene in non-irradiated leaves influences the chlorophyll fluorescence and therefore the photosynthetic activity by retarding it.
In contrast, tobacco leaves treated with GFP gene, which had been exposed to UV, showed a decrease of about 15% of F690/F735 ratio in comparison to the control leaves, as indicated by Table 4-4. These results indicate that GFP increases the photochemical process and therefore photosynthetic activity in UV irradiated leaves. Another possible explanation could be that the presence of GFP protects the leaf to some extent from UV damage to the photosynthetic process. This would result in an increased F690/F735 ratio. For GFP treated leaves the increase in the F690/F735 ratio caused by UV irradiation is only about 2% (1.65 to 1.69), whereas the increase for the control leaves is about 28% (1.55 to 1.98) as indicated by Table 4-4.

The variation of the chlorophyll fluorescence of tobacco leaves subjected to constant illumination at different power levels was analysed. This was done for both leaves with GFP treated and control leaves. Figure 4-15 shows a decrease of the chlorophyll fluorescence F690/F735 ratio for the control leaves. It indicates a decrease of the F690/F735 ratio with increasing power output of the He-Ne laser. The red curve shows the decrease of the chlorophyll fluorescence ratio of control leaves irradiated with a power of 5 mW whereas the green and blue curves represent those with powers of 4 mW and 1.6 mW respectively. The ratio of chlorophyll fluorescence decreases for power levels of 5 mW but remains higher compared to those of 4 mW and 1.6 mW. For longer periods of time (>100 µs) the ratio converge toward a coinciding steady state.

As it is known from the theory of fluorescence that fluorophores have different degrees of fluorescence, and hence the ratio of their fluorescence amplitudes varies with the laser power. Thus the ratio of the red to far red fluorescence decreases with a change of the laser power.

The decrease of the F690/F735 ratios for tobacco leaves treated with GFP as shown in Figure 4-15 exhibit the same characteristic as those of the control leaves discussed above. But these decrease ratios converge toward the steady state significantly faster. The ratio difference is almost indistinct after 20 seconds of irradiation for all three power levels of He-Ne laser used. When the GFP is inserted into tobacco leaves, the ratio of red and far red chlorophyll fluorescence decreases in the same manner for all power levels used. The presence of the GFP gene influences the ratio of the chlorophyll fluorescence bands, as is shown by Figures 4-15 and 4-16, indicating different behaviour of the ratio according to the state of the leaves and the power used.

This experiment has shown that the F690/F735 ratio of chlorophyll fluorescence decreases
Figure 4-15: F690/F735 ratio variation in function of time and function of power output of the laser. The measurements were done at different spots of control tobacco leaf. He-Ne was used as source of irradiation. Red curve variation of F690/F735 for a power of 5 mW, dark-green curve for a power of 4 mW and blue for power of 1.6 mW.
Figure 4-16: F690/F735 ratio variation as function of time and function of power output of the laser. The measurements were done at different spots of tobacco leaf treated with GFP gene. He-Ne was used as source of irradiation. Red curve variation of F690/F735 for a power of 5 mW, black curve for a power of 4 mW and blue for power of 1.6 mW.
as function of time and laser intensity. The presence of the GFP gene in the tobacco leaf does influence the decrease of that ratio. In the case of the treated leaves, after 20 seconds, the ratios of chlorophyll fluorescence for different irradiation power levels tend to coincide; it takes almost 100 seconds for the control leaves. This shows that the GFP has an influence on the chlorophyll fluorescence ratio. In addition, the decrease of the chlorophyll fluorescence ratio with the use of constant radiation on the leaf can be explained by a stronger quenching of the PS II fluorescence, compared to that of the PS I [1, 12, 18].

4.4.2 Imaging results

The laser induced chlorophyll fluorescence imaging system using the excimer laser at 308 nm as excitation source allows one to detect and quantify the chlorophyll fluorescence of the red and far red band in reflection. A tobacco leaf treated with GFP and a control leaf were imaged together as shown in Figure 4-17. Both leaves were irradiated at the same time, hence the intensity of the laser pulse was the same for both leaves. This was done to avoid the effect of pulse to pulse intensity fluctuations of the excimer laser and to be able to directly compare the fluorescence of the GFP treated tobacco leaf to the control leaf.

The images acquired by the CCD camera were transferred to the computer for further analysis. The images were saved as bitmap files and were displayed in black and white. Once the fluorescence images had been collected and saved in the computer, data processing using Microcal Origin software allowed us to plot the images in false color contours in order to show the distribution of fluorescence intensity more clearly. Chlorophyll fluorescence could be used to distinguish GFP treated leaves from the control leaves. Figure 4-17 presents the images of tobacco leaves recorded in the various fluorescence bands using narrow band interference filters. For each image (a, b, c, and d) the treated leaves were positioned to the left of the control leaves. The fluorescence of the treated tobacco leaves was generally significantly higher compared to the control leaves (Figure 4-17a and b). The intensity of red and far red chlorophyll fluorescence is strongly increased in leaves treated with GFP compared to the control leaves. As the red and far red chlorophyll fluorescence exclusively emanates from the chlorophyll, the GFP may have invaded the chlorophyll or it may have caused an inhibition of activity of reaction centers of the PS II and PS I. It therefore reduced the activity of the photosynthetic system, resulting
in an increase of chlorophyll fluorescence production [1].

The images also show that chlorophyll fluorescence is not uniformly distributed over the leaves; the intensity is strong in certain regions of the leaf and weak in others. Figures 4-17a and b show the disparity of chlorophyll fluorescence distribution. The intensity is high around the center of the treated tobacco leaf and decreases towards the edge of the leaf. The intensity of fluorescence differs in certain sites of the control leaves and GFP treated leaves. A possible reason for this disparity of chlorophyll fluorescence resides in the uneven distribution of chlorophyll over the leaf. The chlorophyll content might be higher in some areas and lower in the others.

The blue and green fluorescence of tobacco leaves was also recorded using the laser induced fluorescence system (see Figure 3-6). The images show that there is no great difference between the blue fluorescence (using the 450 nm narrow band filter) of tobacco leaves treated with GFP and the control leaves (Figure 4-17c). This was also noticed in the case of the green fluorescence (using the 520 nm narrow band filter) (Figure 4-17d). The results show that the presence of GFP in leaves did not have an impact on the blue and green fluorescence, and that the fluorescence intensities are uniform for both leaves. The blue and green fluorescence originates generally from the cinnamic acids and other plant phenolics covalently bound to the ferulic acid of the cell wall. Therefore, according to the results obtained, one may conclude that GFP did not invade those proteins and did therefore not influence the blue and green fluorescence.

In other respects the GFP protein produced by the gene incorporated in tobacco leaves absorbs light at 380 nm or 488 nm and it fluoresces at 509 nm. So the use of an excimer laser at 308 nm may excite the GFP molecules and consequently produce GFP fluorescence. Thus the use of 450 nm and 520 nm narrow band interference filters could not only enable the recording of the blue and green fluorescence of leaves but it also could allow the detection of the GFP fluorescence (in the case of high and broad band of GFP fluorescence intensity, the emitted fluorescence of GFP could overlap with the blue and the green fluorescence). Therefore the fluorescence intensity seen in Figure 4-17c and d might be the combination of the fluorescence of GFP, cinnamic acids and plant phenolics molecules. This may explain the uniformity of the fluorescence imaging obtained in the blue and green filters.
Figure 4-17: Chlorophyll fluorescence images of GFP treated tobacco leaf (left) and control leaf (right). Images were recorded using the excimer laser as source of irradiation and narrow band interference filters: a)690 nm, b)730 nm, c)450 nm, d)520 nm.
Furthermore, the ratio of the red and far red chlorophyll fluorescence is an indicator of plant photosynthesis activities and plant stress. Using the chlorophyll fluorescence imaging technique, an image ratio process was performed to measure the ratio of red and far red chlorophyll fluorescence, as described in Section 3.2.2. Figure 4-18 shows the F690/F735 ratio image of the chlorophyll fluorescence. This image (obtained from the ratio of the image of Figure 4-17a over the one of Figure 4-17b) shows the non-uniformity of the ratio over the leaves as a result of the non-uniform distribution of the chlorophyll fluorescence. The ratio is high in some areas and low in the others. This is noticed for the treated tobacco leaf and for the control leaf. In addition, there are more areas showing a high ratio in the control tobacco leaf (Figure 4-18, on the right) than the one treated with GFP (Figure 4-18, on the left).

The chlorophyll fluorescence images were taken after a couple of laser shots (four shots). Therefore leaves can be considered as irradiated before the image is recorded. The image ratios of irradiated tobacco leaves show that control leaves have a higher ratio value than leaves treated with GFP. This is in agreement with the previous results from spectral measurements on irradiated leaves given in Table 4-4.

The camera used to record the fluorescence images was placed at a certain angle with regard to the incident beam of the laser. Therefore the question of angular dependency of the intensity arose. The fluorescence observed in the obtained images might have been influenced by the position of the leaves and the angle at which the recording system was placed. To remove this uncertainty, the position of the leaves was switched, moving tobacco leaves treated with GFP gene to the right and the control leaves to the left. The image of the red chlorophyll fluorescence was then recorded. Figure 4-19 shows the obtained image. The image is in agreement with results obtained previously for the red chlorophyll fluorescence band (Figure 4-17a). A high chlorophyll fluorescence intensity of the red band is still exhibited in tobacco leaves treated with GFP while a low chlorophyll fluorescence is found in the control leaves.
Figure 4-18: Imaging ratio of F690/F735. On the left is the ratio of the tobacco leaf treated with GFP and on the right is the ratio of the control leaf. Images 4-17 a and 4-17 b were used to calculate the ratio.
Figure 4-19: Chlorophyll fluorescence image of the red band, 690 nm. Treated and control leaf interchanged (control leaf left; treated leaf right).
Chapter 5

Summary, Conclusions and Recommendations for Future work

5.1 Summary and conclusions

This work was focused firstly on chlorophyll fluorescence spectroscopy and imaging experimental setups with emphasis on analysis techniques of the spectra and images recorded. Secondly it was devoted to investigate the effect of green fluorescence protein on chlorophyll fluorescence, therefore on the photosynthetic process.

Two detecting systems were developed for chlorophyll fluorescence spectroscopy and imaging techniques. They permit the measurement of the fluorescence intensity of plant materials using XeCl excimer, argon ion or He-Ne laser as excitation source. The use of long pass filters (in spectroscopy system) and narrow band filters (in imaging system) allowed the discrimination between the scattering of the laser and the chlorophyll fluorescence. For the imaging setup, the use of a delay/pulse generator in order to trigger the camera, an A/D card in the computer and the laser was necessary and essential to synchronise the recording system and control the intensity and the sensitivity of recorded images. The developed data acquisition system was found to function very well, allowing the recording of chlorophyll fluorescence spectra and fluorescence images. The results prove that the spectroscopy and imaging technique (using 308 nm, 455 nm and 632.8 nm as excitation wavelengths) can be successfully used to record the chlorophyll fluorescence spectrum and its variation with time and to image chlorophyll
fluorescence in leaves and fruit.

In other respects, the analytical techniques of spectra and imaging process methods were developed in order to extract information of spectra and images. Gaussian fit deconvolution of the chlorophyll fluorescence spectrum allowed the determination of the peak heights, the full width at half maxima (FWHM) and the areas under the red and far red peaks. Results of the determination of the peak heights was used to calculate the F690/F735 peak height ratio, which is an indicator of the state of the leaves and therefore the physiological state of a plant. While fluorescence spectra could be recorded, the spectroscopy system was subject to the problem of changes in fluorescence intensity when measurements were taken on different spots of the leaf or for different leaves in the same state. To overcome the problem of intensity variation, the F690/F735 was calculated. It was found that despite the fluorescence intensity variation the F690/F735 ratio showed only a small variation, which might be negligible (Section 4.1), and the width of the red and far red peaks seemed to remain constant over the whole leaf.

Results on fluorescence quenching were obtained when a leaf was irradiated with a CW laser, and the fluorescence decreased exponentially with time. This decrease was fitted by a second order exponential decay function, and parameters $\tau_1$ and $\tau_2$ which indicate the time constants of the fast and the slow processes in photosynthetic activity were determined. The values of these parameters depended on the source of excitation used. The results in Section 4.2.2 demonstrated that parameters $\tau_1$ and $\tau_2$ changed under different conditions of excitation (argon ion laser at 455 nm and He-Ne laser at 632.8 nm, both with output power of 5 mW).

A single leaf point measurement gave information about a particular leaf point but not of the whole leaf with all its different parts, that usually have different information. A single leaf point measurement was found not to be representative for the whole leaf. However, the fluorescence imaging technique provided information on all irradiated leaf parts and showed differences in chlorophyll fluorescence yield. A number of image processing procedures were implemented by means of software and were successfully used to facilitate the interpretation of the images recorded. The image cleaning procedure facilitated an effective localisation of the regions of high and low fluorescence intensity. In addition it was demonstrated that the method of delaying the camera gate in respect to the laser pulse can be used to discriminate the laser scattering from the fluorescence of the plant material. Using this method one may be able to
determine the fluorescence lifetime of plant material.

This study also explored the possibility of detection of the effect of Green Fluorescence Protein on chlorophyll fluorescence using excimer, argon ion and He-Ne lasers. The 308 nm excitation light of the excimer laser does induce GFP protein fluorescence and chlorophyll fluorescence. Furthermore, the fluorescence peaks at 690 nm and 735 nm represent only chlorophyll fluorescence. This study shows that the fluorescence imaging system with 308 nm excitation can be used to monitor the presence of GFP gene in leaves by recording the chlorophyll fluorescence. It was found that, by imaging the red as well as the far red chlorophyll fluorescence peaks, the leaves treated with GFP gene exhibit a higher chlorophyll fluorescence intensity compared to the control ones, which is an indicator of the influence of GFP gene on chlorophyll fluorescence. In addition, blue and green fluorescence imaging of tobacco leaves were recorded but it was not possible to differentiate between treated and control leaves. The fluorescence intensity was similar for both leaves.

The spectroscopy setup, using the argon ion or He-Ne laser, was suitable to record fluorescence spectra of the tobacco leaves. Looking at the intensity of the chlorophyll fluorescence spectra, it was not possible to distinguish the influence of GFP genes on chlorophyll fluorescence. Nevertheless, the use of the ratio of the red and far red peak heights (F690/F735) allowed the determination of the effect of GFP in tobacco leaves. The ratio was higher for the treated leaves (1.65) than the ratio for the control leaves (1.55). However, it was discovered that when leaves are subject to previous radiation (UV radiation) the ratio changes. The ratio becomes higher for the control (1.98) than the treated leaves (1.69). The present results indicate that it was not only the GFP gene that influenced the fluorescence of tobacco leaves but also the UV light. According to the inverse proportional relation between chlorophyll fluorescence and the photosynthetic process it was concluded that the GFP and UV light have an effect on photosynthetic activity. Both seem to decrease the photosynthetic activity; but if the GFP is present, then UV radiation has a smaller effect.

In this study it was demonstrated that the spectral behaviour of the measured chlorophyll fluorescence depends to some extent on the plant species, as indicated in Section 4.1. The Ligustrum japonicum leaves had a red fluorescence peak (690 nm) that was lower than the far red peak (730 nm), while the tobacco leaves presented a different shape; the far red peak was
lower compared to the red one. Results of laser induced chlorophyll fluorescence under constant irradiation indicate that chlorophyll molecules are quenched, and consequently the fluorescence intensity decreases when the leaf is re-irradiated at the same region. As result of irradiation the photosynthetic process becomes faster, reducing the amount of energy irradiated as fluorescence. The leaf cannot recovery totally in a time period of 5 minutes when it was previously subjected to irradiation. A relation between the laser output power and the fluorescence intensity was established. The fluorescence increases with an increase in laser output power, as demonstrated in Sections 4.2.2 and 4.4.1 (He-Ne at 632.8 nm with output powers of 1.6 mW, 4 mW and 5 mW). It was found that this relation depend on the status of leaves. The tobacco leaves treated with GFP showed a different trend, with the fluorescence intensity remaining constant with increasing laser power.

5.2 Recommendations for Future studies

The imaging system may be used to detect the total fluorescence, but the sensitivity must be improved in order to detect wavelength specific fluorescence. This may require suitable filters and the use of further image intensification. In addition, suitable gating delays have to be determined for each application. Moreover, the cleaning procedure is useful to obtain a clearer image.

A useful extension of this work would be to improve the detecting system of chlorophyll fluorescence spectroscopy by developing a more consistent recording system that limits the fluctuations of fluorescence intensity and will allow better spectral analysis. For an imaging system, an improvement of the image intensification and sensitivity is required. These implementations will allow one to investigate, in detail, the effect of some stressors, not only on chlorophyll fluorescence bands but also on blue and green bands of plant fluorescence. Despite the relation between chlorophyll fluorescence and photosynthetic activity, the effect of GFP genes on photosynthetic process is not totally conclusive. It needs further studies, for example, by investigating CO$_2$ assimilation by the photosynthetic mechanism.
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