Development of a Fluorescence Imaging System for the Quality Assessment of Fruits and Vegetables

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MASSIMO CISCATO

Promotor : Prof. dr. R. Valcke

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Samenvatting

De chlorofyl-fluorescentie technologie is een zeer krachtig werktuig om de functie van het fotosynthese-apparaat in fotosynthetiserende organismen te bestuderen. Het is een zeer gevoelige techniek waardoor de interpretatie van de resultaten echter niet steeds voor de hand ligt. Eén van de grote voordelen daarentegen is dan wel dat het een niet-destructieve techniek is die zeer geschikt is om een snelle screening van een groot aantal samples uit te voeren.

Traditioneel wordt de chlorofyl-fluorescentie gemeten door middel van speciaal ontworpen fluorimeters. Deze toestellen zijn echter gelimiteerd doordat de metingen beperkt bijven tot kleine oppervlakten van het plantenmateriaal (bladeren, vruchten). In het laatste decennium werd een nieuwe benadering ontwikkeld, de chlorofyl-fluorescentie-beeldanalyse. In deze methode wordt de distributie van de fluorescentie-emissie van het chlorofyl, aanwezig in het plantenmateriaal, gemeten in twee dimensies. Het onderzoek in dit domein staat als het ware nog in de kinderschoenen; een aantal onderzoeksgroepen gebruiken verschillende benaderingen om het probleem te bestuderen.

Het voorgelegde werk beschrijft het chlorofyl-fluorescentie-beeldanalyse systeem dat ontworpen en ontwikkeld werd in het laboratorium Plantkunde van het Limburgs Universitair Centrum en de toepassing van deze methode voor de bepaling van de kwaliteit van pitfruit (bv. appelen), voor de studie van de effecten van zware metalen op de plant, het effect van een verstoring van de endogene fytohormonenbalans en als diagnostische methode voor de pre-symptomatische detectie van virale infecties.

Het chlorofyl-beeldanalyse systeem is opgebouwd uit een excitatie-eenheid, een beeldcapterende eenheid en een controle-eenheid. De chlorofyl-fluorescentie wordt geïnduceerd door excitatie met xenon-lampen. Het licht wordt gefilterd doorheen een verzadigde kopersulfaat-oplossing die fungeert als een lage-band pass cut-off filter. De detectie gebeurt door middel van een CCD-camera voorzien van een rood cut-off filter. In dit werk wordt een gedetailleerde beschrijving van het systeem gegeven en relevante technische
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aspecten worden toegelicht. Er wordt speciale aandacht geschonken aan de voor- en nadelen die impliciet in de voorgestelde technische oplossingen vervat liggen.

De voornaamste toepassing van het chlorofyl-beeldanalyse systeem die in dit werk behandeld wordt is in het domein van de kwaliteitsbepaling van pitfruit. Eerst wordt het fluorescentie- emissie-gedrag van een appel, gemeten door middel van de fluorescentie-beeldanalyse, beschreven. Vervolgens worden resultaten van verscheidene experimenten bediscussieerd. Het gebruik van de fluorescentie-beeldanalyse om de ontwikkeling van fysiologische afwijkingen tijdens de opslag van appelen te voorspellen, wordt geïllustreerd aan de hand van een succesvol predictie-experiment. In experimenten op appelen die gekweekt werden met en zonder supplementaire stikstofvoeding tijdens de voor-oogst periode, wordt aangetoond dat de fluorescentie-beeldanalyse, verschillen in fluorescentie-emissie gerelateerd aan de stikstofbehandeling, kan detecteren. In het licht hiervan worden nieuwe perspectieven besproken alsmede de vooruitgang die geboekt wordt in het gebruik van geavanceerde analysetechnieken zoals neurale netwerken (artificiële intelligentie). In een experiment waarin boomgaardener onderworpen werden aan verschillende behandelingen van waterstress konden tot op heden geen merkbare wijzigingen in de fluorescentie-emissie door middel van de chlorofyl-beeldanalyse gedetecteerd worden.

De fluorescentie-beeldanalyse systemen kunnen ook toegepast worden in andere domeinen van het plantenfysiologisch onderzoek. In dit werk worden drie toepassingen belicht: (i) behandeling van planten met zware metalen, (ii) virus-geïnoculeerde *Nicotiana bethamiana* planten en (iii) transgene *Pssu-ipt Nicotiana tabacum* planten met een verhoogd endogene cytokinine-inhoud.

Planten die behandeld werden met zware metalen vertonen een variatie in de fluorescentie-emissie tijdens deze metaalbehandeling. Niet alleen de intensiteit van de fluorescentie-emissie als dusdanig wijzigt zich, maar ook de distributie ervan over het blad. Dit laat toe mogelijke correlaties te leggen tussen de waargenomen effecten en de verdeling van het zware metaal in de plant zelf.

De inoculatie van *Nicotianan benthamiana* bladeren met 'pepper mild mottle virus' induceert een wijziging in het fluorescentie-emissie patroon in de asymptomatische bladeren en dit reeds enkele dagen na inoculatie. Het fluorescentie-patroon dat wordt waargenomen
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kan gerelateerd worden met de verspreiding van het virus vanaf de inoculatieplaats naar de andere delen van de plant.

Transgene Pssu-ipt tabaksplanten, gekenmerkt door een verhoogde concentratie aan endogene cytokininen vertonen in de gebieden van het bladmesofiel, distaal gelegen van de hoofdnerven, specifieke fluorescentie-emissie patronen met gewijzigde kinetieken. Mogelijke verklaringen voor deze fenomenen worden voorgesteld.

De software tools die in dit werk gebruikt werden worden gepresenteerd in de appendices. De volgende pakketten worden beschreven:

- *Grabix*: geschreven door de auteur zelf, controleert het volledige chlorofyl-fluorescentie-beeldanalyse systeem;
- *KhorosPro 2001, Student Edition*: dit is een zeer flexibel softwarepakket dat uitermate geschikt is voor beeldanalyse en wordt gebruikt voor de bewerking van de fluorescentie-beelden na captatie door Grabix;
- *R*: de programmeertaal voor statistische analyse van gegevens en om statistische informatie uit de beelden te extraheren;
- *Stuttgart Neural Network Simulator (SNNS)*: de simulator gebruikt voor de analyse van fluorescentie-beelden door middel van artificiële neurale netwerken.
Summary

Chlorophyll fluorescence has been used for a long time as a powerful tool to investigate the functioning of the photosynthetic apparatus of photosynthetic organisms. It is an extremely sensitive technique, though such sensitivity often makes its results very difficult to decipher. One of its main advantages is that it is a non-destructive technique, which makes it ideally suited for fast screening of large collections of samples. Traditionally, chlorophyll fluorescence is measured by specially designed fluorimeters, which perform point measurements on the sample surface. In the last decade a new approach, chlorophyll fluorescence imaging, has been developed in order to measure the distribution of chlorophyll fluorescence emission in two dimensions. Research work in this area is still at an early stage, and a few research groups developed different approaches to the problem.

The present thesis deals with the development of a chlorophyll fluorescence imaging system carried out at the Laboratory of Botany of the Limburgs Universitair Centrum and its application in the field of fruit quality assessment, in the study of plant heavy metal stress, on the effects of disturbances of the endogenous phytohormone balance and as a diagnostic method for pre-symptomatic detection of viral infections.

The system is composed of an excitation unit, an imaging unit and a control unit. Chlorophyll fluorescence is detected by a CCD camera fitted with a red cut-off filter, upon excitation with xenon lamps filtered with a solution of copper sulphate, which provides a blue cut-off low-pass filter. A detailed description of the system is presented and relevant technical issues are discussed. In particular, advantages and drawbacks implied by the technical solutions adopted are addressed.

The mainstream application of the chlorophyll fluorescence imaging system is in the field of apple quality assessment. After a description of the typical behavior of an apple in terms of fluorescence emission as measured by the fluorescence imaging system, results from various experiments with apple material are reported. The suitability of the technique as a predictor for development of storage diseases is discussed on the basis of an example of successful prediction. In experiments with apples grown with or without extra nitrogen supply, the system proved to be able to detect differences in fluorescence emission related to
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the nitrogen treatment. New perspectives opened by such result are discussed. Progress made in using advanced analysis tools like Artificial Neural Networks is also addressed. The system was used also in an experiment with apples grown in orchards treated with different water regimes. In this case, unfortunately, the system could not detect any remarkable differences related to the irrigation regimes.

The fluorescence imaging systems, though designed with special attention to its application in fruit quality control, can be applied also to other fields of research. A gallery of three examples of an alternative application is presented. In particular, results are shown from experiments made on heavy metal treated bean plants, on virus-inoculated *Nicotiana benthamiana* and on cytokinin-overexpressing transgenic *Pssu-ipt* tobacco.

Heavy metal treated plants showed a variation of fluorescence emission in time during the metal treatment. Not only the intensity of fluorescence emission changed, but also its distribution on the leaf, opening the way to speculations about the possible correlation of the difference observed and the distribution of metal ions within the leaf.

The inoculation of *Nicotiana benthamiana* plants with pepper mild mottle virus caused an alteration of the fluorescence emission pattern a few days after the inoculation in otherwise asymptomatic leaves. A peculiar pattern could be observed, which might be related to the spreading of the virus from the site of inoculation to other parts of the plant.

Transgenic *Pssu-ipt* tobacco, characterized by an elevated content of endogenous cytokinin, also showed a peculiar pattern of fluorescence emission, with altered kinetics in mesophyll areas at a distance from the main veins. Possible explanations of such phenomenon are attempted.

In the appendices, the software tools used in this research work are presented. In particular, a description is given of the following programs:

- *Grabix*: Developed by the author, this is the software that controls the whole fluorescence imaging system.
- *KhorosPro 2001, Student edition*: This is a very flexible multi-purpose software suite for image analysis, used to process fluorescence images after being captured with Grabix.
- *R*: This is the programming language for statistical data analysis used to extract statistical information from the images.
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- Stuttgart Neural Network Simulator (SNNS): It is the simulator used to analyze fluorescence images by means of artificial neural networks.

List of Abbreviations

\(AD/DA\) = Analog-Digital/Digital-Analog; \(ANN\) = Artificial Neural Network; \(ASCII\) = American Standard Code for Information Interchange; \(CCD\) = Charge Coupled Device; \(CK\) = Cytokinin; \(FIS\) = Fluorescence Imaging System; \(DAFB\) = Days After Blooming; \(DPI\) = Days Post-Inoculation; \(F_M\) = maximum of fluorescence induction; \(F_v\) = variable fluorescence; \(LED\) = Light Emitting Diode; \(LHC\) = Light Harvesting Complex; \(NIR\) = Near-Infrared; \(NPQ\) = Non-Photochemical Quenching; \(O.D.\) = Optical Density; \(PEA\) = Plant Efficiency Analyser; \(PGM\) = Portable Grey Map; \(PSII\) = photosystem II; \(Q_A, Q_B\) = plastoquinone; \(ROI\) = Region Of Interest; \(R_fd\) = Ratio of fluorescence decay; \(TLC\) = Thin Layer Chromatography; \(UV\) = Ultraviolet.
Chapter 1
Introduction

Chlorophyll fluorescence is a fascinating phenomenon that has attracted the attention of researchers for many decades now. The reasons of its success can be found in its enormous potential as a powerful non-destructive probe to investigate the physiology and structure of the photosynthetic apparatus of photosynthesizing organisms. Moreover, as core element of the physiology of plants (I am using here the term plants in its broadest meaning), photosynthesis is affected by the overall conditions of the organism. As a consequence, its investigation by means of chlorophyll fluorescence turns out to be a powerful tool for the analysis of plant health, with a wide range of applications. This simple non-invasive means of studying photosynthetic electron transfer reactions has been applied in several fields, from basic photosynthesis research (Krause and Weis, 1991) to large-scale field-testing of plant health status by means of easy to use portable fluorimeters (Mohammed et al., 1995). One of the most intriguing applications of this technique is the early detection of physiological disorders and stress, when visual symptoms are not yet present. This subject has stimulated the production of many scientific papers and reviews (Lichtenthaler and Rinderle 1988; van Kooten and Snel, 1990, Lichtenthaler and Miehé, 1997).

The present thesis deals with the development of a chlorophyll fluorescence imaging technique for the quality assessment of fruits and vegetables, and the health status of plants in general. In this chapter, after a brief introduction about chlorophyll fluorescence, the basics of the chlorophyll fluorescence image analysis will be presented. This is followed by an introduction to the concepts of fruit quality, with special attention paid to non-destructive techniques used for its assessment. The fluorescence imaging system will be described in chapter two, where issues concerning specific technical solutions will also be addressed. In chapter three, data resulting from the application of the imaging system to apple material will be presented. Chapter four will introduce other applications of the imaging system. In particular, results from experiments with plants affected by heavy metal stress, viral infections or genetically induced cytokinin-overexpression will be presented. Chapter 5 contains general conclusions about the work presented. Various software tools used in the different phases of
the research work, both commercial products or developed by the author, will be described in
the appendix.

1.1 Chlorophyll Fluorescence

1.1.1 Molecular aspects

Plants, using the available atmospheric transmission bands, absorb light over a broad
spectrum of wavelengths, ranging from the UV to the red region of the electromagnetic
spectrum, thanks to a complex pool of pigments organized in specific structures in the
thylakoid membranes of the chloroplasts. Absorption properties of pigments can be explained
in terms of their chemical structure.

The reason why chlorophyll and other plant pigments absorb visible light lies in the
physico-chemical properties of their molecules. When an organic molecule absorbs a photon

![Molecule of Chlorophyll a. Left: chemical structure. Right: three-dimensional model.](image)

**Figure 1-1** Molecule of Chlorophyll $a$. Left: chemical structure. Right: three-dimensional model.
of specific wavelength, an electron undergoes a transition from its ground state to a higher
electronic energy level, i.e. from a bonding ($\sigma, \pi$) or non-bonding ($n$) molecular orbital to an
anti-bonding ($\sigma^*, \pi^*$) molecular orbital. Four such transitions are possible ($\sigma \rightarrow \sigma^*, \pi \rightarrow \pi^*, n
\rightarrow \sigma^*, n \rightarrow \pi^*$), all of them requiring photons at wavelengths shorter then those in the visible
region of the electromagnetic spectrum. However, in molecules with two or more conjugated
double bonds, the wavelength of the photons causing the transition $\pi \rightarrow \pi^*$ increases with the
number of such bonds. Molecules with more than seven conjugated double bonds, such as
carotenoids and chlorophylls (fig. 1-1), absorb photons having wavelengths in the visible
region.

The excited state is unstable, and the high-energy electrons tend to fall back to the
ground state. This may happen via several competing pathways, with or without radiation
emission. Fluorescence is the re-emission of part of the absorbed light at a longer wavelength,
which is associated with the drop of one electron from his first- or second-excited-state singlet
back to the ground state. Vibrational cascade can occur at both the ground and excited state,
causing a loss of energy, which explains why a photon is emitted at a longer, and hence less
energetic, wavelength. This process happens on a timescale of $10^{-9} \rightarrow 10^{-5}$ sec. At room
temperature, most of the fluorescence observed in plants is emitted by chlorophyll $a$ of
photosystem II. Under normal physiological conditions, the fluorescence yield of chlorophyll
$a$ is quite low, ranging from 0.6% to 3%; most of the absorbed energy (more than 90%) is
used for primary photochemistry (Krause and Weis, 1991, and cited literature).

1.1.2 Emission spectrum of chlorophyll fluorescence

The fluorescence emission spectrum of chlorophyll $a$ shows two maxima in the far-
red region. Chlorophyll $b$ has a similar spectrum to chlorophyll $a$. However in a concentrated
solution and in vivo chlorophyll $b$ does not fluoresce and its excited singlet state is transferred
100% to chlorophyll $a$ (Lichtenthaler and Rinderle, 1988). Intact leaves only exhibit
chlorophyll $a$ fluorescence. The height and position of the two peaks vary in vivo or in
solution, and depend on the excitation wavelength and pigment concentration, as well as on
the temperature. In the case of a pigment solution, the solvent plays also a role in determining
the shape of the emission spectrum. Figure 1-2 shows emission spectra of chlorophyll $a$
extract in 95% aqueous ethanol at various concentrations. Increasing the pigment
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concentration in solution causes a change in the ratio between height of the short-wavelength peak and high-wavelength peak. This is due to re-absorption of the fluorescence emitted at the shorter wavelength by the chlorophylls, which absorb in the same region (fig. 1-3).

The situation in vivo is much more complicated. The chlorophyll molecules are organized in protein complexes embedded in the thylakoid membrane inside the chloroplast (Hankamer and Barber, 1997). The chloroplasts are positioned inside the cells and the cells are organized in structured layers (fig 1-4). All these factors contribute to create a very complex optical structure, which influences both the absorption and emission of light, as well as the energy transfer between the various components of the photosynthetic apparatus (Volgemann et al. 1996). Upon light excitation, the absorbed energy is transferred from the pigments in the antenna and in the light-harvesting complex (LHC) to the chlorophylls of the reaction centers of the two photosystems.

![Chlorophyll-a in 95% ethanol](image1)

**Figure 1-2** Fluorescence emission spectra of chlorophyll a in 95% ethanol at different concentrations. The numbers beside each curve indicate the concentration on chlorophyll a in μg ml⁻¹. From Gitelson et al. (1998)

![Absorption spectra of chlorophyll a in methanol](image2)

**Figure 1-3** Absorption spectra of chlorophyll a in methanol. Generated by PhotochemCAD (Du et al., 1998).
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Through what kind of path the energy transfer occurs, is still a matter of debate. Several models have been developed. The two main ones are the lake model and the puddle model, as Robinson (1967) named them (fig. 1-5). According to the lake model, the excitons can be transferred randomly within the antenna and eventually reach any reaction center. In the puddle model, instead, each reaction center can receive excitons only from its own

Figure 1.4 Structure of the leaf of dicotyledonous plants. Reproduced from Online Biology Book.

Figure 1.5 Lake and Isolated Puddles models of excitation energy flow in photosynthetic pigment systems. ○ = antenna pigment. ● = P700 or P680 at the reaction center. ▲ = antenna pigment molecule in an excited state having received a quantum of energy by absorption of a photon or by energy transfer. From Goodwin and Mercer, 1983.
antenna pool. As Govindjee (1995) pointed out, the real situation might be a combination of the two models, i.e. a puddle model with some probability of energy transfer between puddles.

Excitation at different wavelengths causes fluorescence emission with peaks at different wavelengths. For example, blue light gives rise to two emission peaks in the red and in the far-red bands, while upon excitation with radiation in the UV-A region four fluorescence emission peaks are induced, two in the blue/green region of the spectrum and two in the red/far-red (fig. 1-6). The photosynthetic apparatus emits not all of the different components of fluorescence.

The emission in the blue/green region, which does not show time dependent variation, is attributed to phenol derivatives (Lichtenthaler and Schweiger, 1998), while the time-variable red/far-red emission is considered to be due to emission by chlorophyll (Lichtenthaler et al., 1996; Strasser et al., 1995). The various components of fluorescence can be used to obtain information about several aspects of the plant physiology (functioning of the photosynthetic apparatus, plant secondary metabolites).

1.1.3 Tissue optical properties

Ultimately, the optical properties of the tissue (leaf or fruit peel) affect the efficiency of harvest and use of light by the plant. Several concurrent phenomena determine the spectral composition and the intensity of the light reaching inner layers of the plant tissue.

At surface level incident light is partly reflected by the leaf or fruit. Part of this light comes from the cuticle/air interface, and part comes from inner layers, where the light rays
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Figure 1-7 Cross section of a Bladder Saltbush (Atriplex vesicaria) leaf illustrating how radiation of different wavelengths may be reflected and absorbed. (a) Fluid-filled trichomes; (b) Epidermal cells; (c) Elongated hypodermal cells; (d) Chloroplasts; (e) Palisade cells; (f) Bundle sheath cells; (g) Oxalate inclusion.

encounter other air/cell interfaces and are reflected. The light reflected from the cuticle usually doesn't change spectral composition, and it is often polarized, while the light coming from inner layers changes spectroscopically depending on the pigment composition of the tissue. Reflection properties of the cuticular surface are also influenced by the presence of epicuticular waxes and trichomes.

Besides reflection, other phenomena come into play in deeper cellular layers. Epidermal cells act as 'cellular lenses' that focus light two to three times or even more (Martin et al. 1989; Vogelmann 1993; Vogelmann et al. 1996). Also, the presence of epidermal hairs can induce the capture of dewdrops, which also act as lenses. As a consequence light is concentrated to levels substantially higher than incident light. Focusing of light in some areas implies that other parts receive less light, causing heterogeneity of the light exposure. Some areas will then receive light irradiation several times more intense than incident light, while other parts will be hit only by a fraction of it. Martin et al. (1989) speculated that some of the chloroplast within the leaf may be adapted to local high light conditions created by the epidermis. Furthermore, light penetrates the tissue to a different extent, depending on its wavelength (fig. 1-7). Radiation with a shorter wavelength may penetrate more deeply within the leaf.

To make the picture even more complex, the fluorescence emitted by chlorophyll molecules situated in deeper cellular layers is reabsorbed inside the leaf on its way to the upper layers, before it leaves the surface. This causes the true emission spectra to be different at different depth within the leaf. Gitelson et al., (1998) by combining absorption and
reflectance measurements were able to correct for re-absorption and obtain \textit{in vivo} spectra very similar to those obtained \textit{in vitro} with diluted chlorophyll solutions.

1.1.4 Kinetics of fluorescence emission

Chlorophyll fluorescence exhibits a peculiar kinetics of emission. Upon illumination of a dark-adapted leaf, chlorophyll \( a \) fluorescence is emitted with an intensity that varies in time. Dark adaptation is used in order to completely oxidize the reaction centers and deplete the photosynthetic electron transport of electrons present in the chain. In such conditions, it is possible to gather information about the kinetics of the electron transport. Dark adaptation is usually obtained by placing the sample in a lightproof environment (e.g. a cabinet) or by applying specially suited clips to the leaf for a period varying from about 20–30 minutes to a few hours. Excessive time of dark adaptation should be avoided to prevent chlorophyll breakdown. The duration of the optimal dark adaptation varies with the plant species and ideally should be determined experimentally, case-by-case. In order to do that, specimens from a sample are dark-adapted for a variable time and the fluorescence emission is measured. A sample is considered to be in a fully dark-adapted state when its peak fluorescence emission \( F_p \) (see below) reaches its maximum value \( F_M \). Dark adaptation is an important factor in the measurement of fluorescence emission by living samples. On the one hand, it is paramount having a proper dark adaptation in order to obtain meaningful information on the functioning of the photosynthetic apparatus. On the other hand, applicability of fluorescence technology in real life situations, outside research laboratories, requires finding suitable technical solutions in order to avoid the constraints imposed by the necessity of dark-adapting the

![Polyphasic chlorophyll \( a \) fluorescence transient emitted upon illumination of a dark-adapted sample. From Govindjee (1995).](image)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chlorophyll_fluorescence_transient.png}
\caption{Polyphasic chlorophyll \( a \) fluorescence transient emitted upon illumination of a dark-adapted sample. From Govindjee (1995).}
\end{figure}
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sample. As an example, let us consider fruit sorting lines. Such machineries nowadays work at a rate of on the order of 4 apples per second. If we wished to integrate fluorescence technology into fruit sorting lines, a dark adaptation time of even only a few minutes would be unthinkable. Alternative solutions must be found. In chapter three this matter will be re-addressed in more detail.

The fluorescence emission from a dark-adapted sample (fig. 1-8) starts, upon illumination, from a basic level, called $F_0$, goes up in a polyphasic fashion (Strasser et al., 1995) to a peak level, $F_p$, which is reached in a few hundred milliseconds. If the sample is fully dark adapted, the peak of fluorescence emission is called $F_M$ (maximum, $P$ in the figure). Beyond the peak, the fluorescence emission falls back down to a steady state level, $F_S$ (T in the figure), which is reached within minutes, possibly with the interposition of some local peaks, depending on the physiological conditions of the sample. This phenomenon is known as “Kautsky effect”, after the German scientist who first described it, back in the 30’s (Kautsky and Hirsch, 1931). The two phases, the initial rising up to the maximum and the decay, are often described as “fast induction phase” and “quenching phase”, respectively. They reflect various aspects of the physiology of photosynthesis, and consequently they are affected by different internal and external factors. This makes chlorophyll a fluorescence a very sensitive tool to investigate the physiology of photosynthesis, but also very complex to decipher.

The work presented here is concerned exclusively with the quenching phase of the chlorophyll fluorescence induction. The reason for this is practical, as technical constraints do not permit measuring with the imaging system either $F_0$ or any of the intermediate steps of the fast induction phase. Further details about this matter can be found in chapter two. While the fast induction phase reflects the reactions of primary photochemistry, which lead to the complete closure of the reaction centers, i.e. to their complete reduction of the QA pool (Strasser et al., 1995), the quenching phase of chlorophyll fluorescence can be ascribed to several concurrent phenomena of both photochemical and non-photochemical nature. Careful analysis of the quenching of the variable chlorophyll fluorescence can bring information about the efficiency of energy conversion in the reaction center of photosystem II, as well as about non-photochemical processes (Krause and Weis, 1991).

Over the years, a number of instruments have been developed to measure chlorophyll fluorescence. The various designs approach chlorophyll fluorescence from different
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standpoints, and allow gathering specific information. In the last decade, a new approach in chlorophyll fluorescence studies, the two-dimensional fluorescence image analysis, has given promising results (Genty and Meyer, 1994; Buschmann and Lichtenhale, 1998).
1.2 The Chlorophyll Fluorescence Imaging

Measuring chlorophyll fluorescence emission with conventional instrumentation has some limitations. By ‘conventional’ we mean here fluorimeters equipped with small-sized sensors, which deliver measurements of fluorescence intensity averaged over the area of the sensor. The most popular among such instruments are the P.A.M. (Pulse Amplitude Modulation) series by Waltz (Effeltrich, Germany), and the P.E.A. (Plant Efficiency Analyser) by Hansatech (Norfolk, UK). With that kind of instrument it is possible to collect only information about a small spot, which is rarely representative of the situation in the whole leaf. In section 4.1 an example is given of a typical situation in which use of point-measurements can provide only incomplete information (Ciscato and Valcke 1998). Information about the spatial distribution of fluorescence emission could still be collected.

Figure 1-9 Light scattering within leaf tissue. The beam of a He-Ne laser pointed at the leaf surface causes the red spot in the small square. In the big square the detail is enlarged and the 1 mm diameter spot generated by the laser beam is masked in black.
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with such instruments by performing series of measurements regularly distributed over the surface to be analyzed. However, such an approach, besides being tedious and inefficient, presents some major problems. The resolution of the system is forcibly very low, as imposed by the size of the detector. For example, even using an optic fiber with a square ending of 2 mm size (which on the other end causes problems of sensitivity, due to the small size of the fiber) we could obtain 400 measurements over an area of 16 cm². The imaging system described in the present work allows, in a typical configuration, 90,000 data points over the same 16 cm² area, and with one single measurement. Even higher resolutions are possible. Furthermore, when performing point measurements, problems arise from the light being scattered within the plant tissue. As an example, in figure 1-9 we can see light scattering within a leaf as generated by the beam of a He-Ne laser. The red area surrounding the spot where the laser beam hits the surface, as highlighted in the figure by masking the area hit by beam with a black spot, is due to light scattered within the leaf tissue. When performing the measurement on a point on the leaf surface, part of the excitation light and of the emitted fluorescence is scattered and eventually, hits the cells in neighboring areas. This will influence the light adaptation status of those cells, biasing the fluorescence measurement. For example, if we were performing an experiment in a condition of dark adaptation, we would need to wait for some time for the areas around a point just measured to go back to a fully dark adapted state. Obviously, this is extremely inconvenient from a practical point of view.

Chlorophyll fluorescence imaging allows to overcome some of these problems, giving access to pieces of information otherwise impossible, or at least extremely difficult, to obtain.

In a sense, the very first experiment about imaging chlorophyll fluorescence can be ascribed to Kautsky and Hirsch, who used their own eyes to visually observe chlorophyll fluorescence emission in their work of 1931, often referred to as the first report about chlorophyll fluorescence changes with time of illumination.

In the early days of chlorophyll fluorescence imaging, pictures of fluorescence emission were obtained photographically by a technique called phytofluorometry (Sundbom and Björn, 1977). More recently, the advent of the relatively inexpensive CCD technology, opened new perspectives to the study of the two-dimensional distribution of chlorophyll fluorescence emission. The availability of digital imaging techniques made it possible to
perform quantitative analysis of the fluorescence images, and not only qualitative. As far as technical solutions are concerned, various research groups have used different approaches. Basically, there are two main types of (chlorophyll) fluorescence imaging systems, differing with respect to the light source used to induce fluorescence: laser-based systems, like the FIS system developed at the laboratory of the Groupe d'Optique Appliquée of the CNRS, Strasbourg, France, and systems using lamps, like, among others, the one described in the present work. Recently, a third kind of imaging system, using light emitting diodes (LEDs) and capable of using modulated excitation light, has been developed at the Institute of Microbiology of the Academy of Sciences of the Czech Republic, Třebůň, Czech Republic (Ladislav Nedbal, personal communication).

*Laser-induced fluorescence imaging*

The Strasbourg-Karlsruhe imaging system (Sowinska et al., 1998) uses a pulsed Nd:YAG laser as excitation source. The excitation wavelength is 355 nm. Exciting in the UV allows eliciting fluorescence emission at four wavelengths (440, 520, 690 and 740 nm), which is detected by an intensified and gated CCD camera equipped with a filter. With such system Lang et al. (1994) could observe an uneven distribution over the area of tobacco leaves of not only the chlorophyll fluorescence, but also of the blue green fluorescence, which is emitted mainly by ferulic acid covalently bound to cell wall carbohydrates (Morales et al., 1996; Lichtenhaler and Schweiger, 1998). Later, the system has been applied in a variety of situations, mostly related to vegetation stress (Lang et al., 1995; Lichtenhaler et al., 1995; Lang et al., 1996; Heisel et al., 1996; Lichtenhaler et al., 1997). The authors were able to show that laser-induced fluorescence images and ratio images obtained by rationing images of the four bands, allow the early detection of stress conditions in plants. Laser-based imaging systems work with a very low light intensity, less than a fraction of one μE s⁻¹ m⁻², and detect steady-state fluorescence. This is not the case with most of the incandescent-lamp-based systems.

*Visible-light-induced fluorescence imaging*

Incandescent lamps can be used to excite the sample. This allows reaching saturating or nearly saturating light intensities over relatively large areas. It is then possible to gather information on the kinetics of fluorescence emission, which reflects the physiology of the photosynthetic apparatus.

One of the first computerized chlorophyll fluorescence imaging systems was the one
developed by Omasa and coworkers (Omasa et al., 1987), who followed chlorophyll fluorescence induction of sunflower and cucumber leaves under relatively low light excitation.

Daley and coworkers (Daley et al., 1989) were able to analyze the topography of photosynthetic activity of leaves with a system, which recorded fluorescence emission in time on a magnetic videotape. Digitization of the images was carried out successively. More recently, the same authors applied their system to plant samples subjected to various stress factors, showing how stress conditions cause spatial and temporal heterogeneities in the chlorophyll fluorescence emission and photosynthetic activity (Raschke et al., 1990; Osmond et al., 1998, Osmond et al., 1999). In particular, they have investigated the effects of viral infections on photosynthesis (Osmond et al., 1990; Balachandran et al., 1994).

Fenton and Crofts proposed a relatively inexpensive solution for a chlorophyll fluorescence imaging system (Fenton and Crofts, 1990), suggesting its use as a tool for the screening of photosynthetic mutants.

One of the most active research groups involved in chlorophyll fluorescence imaging is the one directed by Bernard Genty in Paris. Based on the idea that relative changes of the quantum yield of the linear electron transport can be quantified by calculating the parameter $\Phi_{PS2}=1-\Phi/\Phi_M$ (where $\Phi$ and $\Phi_M$ are relative fluorescence yields measured at steady state) (Genty et al., 1989), they have built an imaging system capable of visualizing the spatial distribution of leaf photosynthesis (Genty and Meyer, 1995). It does so by imaging fluorescence at the steady state and during a saturating light pulse. The two images obtained are then used to calculate a third image representing the spatial distribution of the abovementioned parameter, and hence of the quantum yield of photosystem II. The system has been used to investigate the role of stomatal conductance in the regulation of photosynthesis (Bro et al., 1996; Meyer and Genty, 1998; Meyer and Genty, 1999). In the same field, Cardon et al. (1994) have investigated the dynamics of patchy stomatal movements in Helianthus and Xanthium by means of fluorescence imaging.

The system developed by Rolfe and Scholes (1995) has been used by the authors to study the effect of fungal pathogens on photosynthesis (Scholes and Rolfe, 1996). The same field has been investigated by means of chlorophyll fluorescence imaging also by Peterson and Aylor (1995). Ning et al. also used fungal infection as a sample application to describe their fluorescence imaging system (Ning et al., 1995).
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Other works involving chlorophyll fluorescence imaging are those of Siebke and Weis (1995) who imaged photosynthetic oscillation in leaves of *Glechoma hederacea*, and Oxborough and Baker (1997a,b) who focused their attention in resolving quenching components from the fluorescence images.

In the field of fruit quality assessment, the application of chlorophyll fluorescence imaging has still been very limited (Abbott, 1996; Abbott 1999). The present work is the first extensive research aimed to apply chlorophyll fluorescence imaging techniques to apple fruit quality assessment.

1.3 Chlorophyll Fluorescence and Fruit Quality Assessment

Grading the quality of agricultural products and fruit in particular is very important at all levels of the production chain, from the orchard down to the end-consumer. Defining quality in this context is not an easy task, as quality estimate is often bound to personal judgment. A certain number of physical properties of fruit, however, are recognized to play a role in the identification of a quality product. Size, shape, color, flavor, texture, taste, they all contribute to the identification of a desirable product by the end consumer, who is the final evaluator of the quality of a fruit. Many methods have been developed over the past decades for quality sorting (Abbot, 1999; Chen, 1996).

The time of harvest of apple fruits is probably the most critical factor in the production of high quality apples. The stage of maturity at which the apples are harvested has an influence on the storage properties of the fruit. Early or late harvest can lead to storage disorders. Harvesting too early can result in poor quality fruits (small, poorly colored, off-flavor) subject to bitter pit and storage scald. Harvesting too late can cause development of watercore and produces fruits with shorter storage life. Being able to estimate the correct harvest maturity of the fruit is of the highest importance in order to schedule a correct time for the harvest. It is worth noting at this point that harvest ripeness is different from market ripeness. The former indicates the maturity level at which the fruit is ready to be harvested for long-term storage; the latter indicates the maturity level at which the fruit is ready for consumption.

Most of the techniques in use nowadays to estimate maturity are based on destructive practice and rely heavily on personal judgment and experience. The number of days after blooming (DAFB) can be used as a general indicator for a candidate period for the
harvest. DAFB values vary greatly among cultivars and can vary also from year to year. Table 1-1 reports estimated DAFB indices for some apple cultivars (adapted from: Pennsylvania Tree Fruit Production Guide, 2000). Tests that are routinely performed to estimate apple maturity include fruit firmness, seed color, sugar levels, starch levels, acid levels and fruit texture.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Bloom</th>
<th>Harvest</th>
<th>Range est. DAFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delicious</td>
<td>Midseason</td>
<td>Late Sept.</td>
<td>135-155</td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>Midseason</td>
<td>Mid Sept to early Oct</td>
<td>135-150</td>
</tr>
<tr>
<td>Granny Smith</td>
<td>Late season</td>
<td>Early Nov</td>
<td>165-180</td>
</tr>
<tr>
<td>Jonagold</td>
<td>Midseason</td>
<td>Late Sept</td>
<td>135-150</td>
</tr>
<tr>
<td>Jonathan</td>
<td>Midseason</td>
<td>Mid to late Sept</td>
<td>135-145</td>
</tr>
</tbody>
</table>

Table 1-1 Estimated days after blooming (DAFB) indices for some apple cultivars (adapted from: Pennsylvania Tree Fruit Production Guide, 2000).

Fruit firmness can be measured by means of suitable penetrometers; repeatability of the measurements is not always granted, as the result is influenced by the *modus operandi* of the operator. Seed color is a quite reliable indicator; this test is based on the fact that the seed color changes from white to dark-brown/black as the fruit ripens. The percent soluble solids (or sugar levels) test is based on the measurement of the percentage of sugar present in the fruit. It is performed on juice samples by means of a refractometer. The sugar is the result of the conversion of starch upon maturation. Measuring the starch content is indeed another technique to estimate fruit ripeness. The measurement is made by applying iodine solution to a cut sample. The fruit flesh gets blue-colored with a specific pattern depending on the starch content. As fruit ripens, starch levels decrease and sugar levels increase. A classification and an estimate of the maturity level are possible according to standards. Acid levels are used to estimate maturity, because the acid content declines as the fruit ripens. Simply chewing a bite of the fruit usually does the testing for fruit texture, and it is a highly subjective test. All of these are destructive methods. The analysis of the peel color is probably the only non-destructive technique routinely used to estimate fruit maturity.

Interest is ever growing concerning non-destructive techniques (Chen and Sun, 1991). Many of the non-destructive techniques are based on one or more of the physical properties mentioned at the beginning of this section, but the most practical and successful among them are based on the optical properties of the product. The characteristics of the
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radiation that leaves the surface of a fruit depend on the incident radiation but also on the properties of the fruit. From the study of reflectance, transmission and emission (fluorescence, phosphorescence and delayed-light emission) that leave the surface of a fruit it is possible to collect information related to the quality of the fruit (Chen and Sun, 1991).

Using time-resolved reflectance spectroscopy Cubeddu et al. (1999) were able to assess internal optical properties of apple fruits, and speculated on the possibility to correlate their results with firmness, one of the most important quality parameters. Near-infrared (NIR) spectroscopy of apples has been correlated by Peirs et al. (1998) to physiological properties of the fruit like maturity age, soluble solid content, starch content and acidity.

Attempts have been made also to correlate chlorophyll fluorescence emission to physiological properties of the fruits in order to estimate maturity/senescence (Song et al., 1997), to predict development of diseases (DeEll et al., 1996) or to detect stress conditions during storage (DeEll et al., 1995). Though those results were encouraging, the experimental approach used suffered from the constraints imposed by the use of conventional instrumentation to measure chlorophyll fluorescence. By ‘conventional’ we mean fluorimeters equipped with small-sized sensors, as compared to the fluorescence imaging.

The present work is a first step in establishing chlorophyll fluorescence imaging as a tool to help estimating quality of agricultural products.

It is however necessary to stress that in general only a combination of techniques will prove to be adequate in order to achieve a reliable estimate of the quality of a fruit or vegetable. Also, overall quality is often not a linear combination of the measured parameters, which poses extra complications for the determination of the best combination of parameters to be used in grading the product. Advanced data analysis techniques, like those based on Articial Neural Networks, may help sort through these multi-parameter datasets.
Chapter 2

The Fluorescence Imaging System (FIS)

This section describes the present configuration of the fluorescence imaging system. The system, the result of an ongoing process of further development, in its current implementation is fully functional. Data presented in chapters three and four where collected with the system in its present configuration. In this chapter, after an overview of the instrument, a detailed description of the main components is presented. Details of specific technical solutions, together with a discussion of related constraints, are presented in separate subsections.

2.1 FIS Overview

Figure 2-1a shows a picture of the fluorescence imaging system developed at the laboratory of Botany of the Limburgs Universitair Centrum. Figure 2-1b shows simplified schematics of it. The system consists of an excitation unit, an imaging unit and a control unit. The sample is laid on a xyz-adjustable ring-shaped holder enclosed in a lightproof cabinet. A CCD camera and the distal end of optic fiber arms are also mounted inside the cabinet. The system is fully automated and computer-controlled.

The light provided by the excitation unit induces the chlorophyll fluorescence of the sample, which is detected by the imaging unit. Successively, digital image analysis is performed to retrieve physiologically meaningful information.

Figure 2-1 Top (a) the fluorescence imaging system. Bottom (b): schematics of the system.
2.2 The Excitation Unit

2.2.1 Description

The excitation unit has been designed to allow illumination of the sample over a large area with two different light intensities: a lower one (Actinic) and a higher one (Saturating). The two different light intensities are needed in order to discriminate components of the fluorescence quenching.

Actinic light is provided by a 150 W xenon lamp, model FOT 150 FiberOptic P. +P. AG, Spreitenbach, Switzerland, and brought inside the cabinet by a flexible, 1 m long, 1.3 cm core diameter, randomized and steel-tipped armored glass fiber-optic bundle. Inside the lamp house, heat from the lamp is greatly reduced by a standard heat reflecting and absorbing optical filter. On the distal end of the fiber bundle a cut-off low-pass filter is mounted. Its function is to provide blue actinic light over a large area when the experiment requires this. Figure 2-4 shows its transmission spectrum (light blue line).

Saturating light is provided by four additional 250 W xenon-lamps (Osram Xenophot HLX, Osram GmbH, München, Germany) each mounted in a lamp housing with heat filter assembly (LQ 2600, Fiberoptic-Heim AG, Uetikon am See, Switzerland) and equipped with flexible, steel-tipped, armored, bifurcated, step-index fiber-optic bundles (model LMA 9/2, Spindler & Hoyer, currently Linos Photonics AG, Goettingen, Germany), with a 9 mm core bundle diameter.
Chapter 2 - The Fluorescence Imaging System

Figure 2-4 Transmittance spectra of the filters used in the fluorescence imaging system.

The glass fiber body effectively shields any spurious UV light that might pass through the excitation filter.

A specially designed quartz bottle containing a saturated solution of CuSO₄ (fig 2-2) was placed in the light path inside the housing of each lamp (fig 2-3); a loosely attached aluminum bottle cap prevents rapid evaporation and build-up of overpressure. Such arrangement, besides cooling the light beam, provides filtered visible light of <650 nm to illuminate the sample. The idea of using a copper filter comes from the chlorophyll fluorescence imaging system of Fenton and Croft (1995). Its transmission spectrum is reported in figure 2-4 (dark blue line). Eight fiberoptic arms are mounted inside the cabinet on angle-adjustable, spring-loaded clamps attached to a metal ring of 10 cm (fig 2-5), which can be placed at a variable distance from the sample to adjust the illuminated area. A close up of the fiberoptic-ring-system is shown in figure 2-6. The optical arrangement of the fiber bundles allows illumination over a circular area of a size depending on the distance from the sample. Light intensity over the sample also depends on the distance of the fiberoptic terminations from the sample.
In the most typical arrangement the distance of the optic fibers from the sample is 12 cm, allowing a circular area of 10 cm diameter to be illuminated by intense light. Under these conditions, actinic light intensity level has a maximum of approximately 220 μE m⁻² s⁻¹ at the center of the illumination field and decreases to about 50% on the edge, while saturating light intensity has a maximum of approximately 1100 μE m⁻² s⁻¹ at the center of the field and also decreases to about 50% at the periphery. Such light intensity is a good compromise between the need for saturating levels of photon flux density and the necessity to image a surface as wide as possible. The spatial arrangement of the system allows keeping the intensity variations across the light field within acceptable limits. Figure 2.7 shows a cross section of the spatial distribution of the light intensities (actinic and full) over the imaging field, with the fibers at a distance of 12 cm from the sample as measured by the camera (left) or by means of a quantum meter (right) model LI-189 (LI-COR, Inc. 4308 Progressive Avenue Lincoln, NE, USA). The values are expressed in relative units normalized to the maximum value of the light intensity. The difference in radial response of the actinic light intensity as measured by the two
methods can be ascribed to geometrical effects related to effective sensor head illumination. Variation of the intensity across the illumination field is corrected for during image processing (see section 2.5 for details). Also, typically only a square region at the center of the image is extracted for analysis, where the intensity variation across the area is only about 10%.

The blue glass and the copper filters are used in combination with a red cut-off high-pass filter mounted on the camera lens (see section 2.3 for details) to avoid overlap between the emission and excitation spectra (fig. 2-4). Such arrangement is required in order to grant imaging of only fluorescence and to completely exclude any light reflected from the sample surface.

Figure 2-7 Intensity profiles over the illuminated area. Left, as measured by the fluorescence imaging system (data extracted from the images). Right, as measured by a quantum meter. Top, horizontal profile. Bottom, vertical profile. Intensity values are expressed as relative to the maximum. X-axis units are pixels for the charts on the left and centimeters for the charts on the right.
2.2.2 Technical Issues

The illumination system is the most demanding part of the apparatus. Close attention has to be paid to the necessity to filter the light in order to avoid imaging any reflected light, and to the difficulty of reaching high levels of uniform light intensity over a wide area.

Using a liquid copper sulphate filter dramatically reduces the light intensity delivered by the lamps. Also, using fiberoptic light-guides results in a limited light intensity at the distal end of the fiber, though the bundles we use are among the thickest available on the market. The use of fiberoptic bundles, however, is unavoidable in order to create a confocal arrangement for the illumination light source and the camera.

In order to guarantee a sufficient excitation light intensity over a relatively large area, a compromise needs to be found by adjusting the distance of the illumination ring from the sample. In our setup we have privileged the area size over the light intensity. Other authors made the opposite choice, limiting the size of the area imaged by their systems (Genty and Meyer, 1994; Cardon et al., 1994). We are aware that this choice of ours poses problems at the level of interpretation of the physiology of the observed phenomenon. However, the possibility to image large areas, allows monitoring fluorescence at the level of the entire leaf, which represents a novel approach.

The use of alternative light sources like, for example, light-emitting diodes has been attempted, but the results in terms of light intensity and homogeneity where not satisfactory. Besides, the excitation light obtained with the copper sulphate filter has a broad spectrum as compared to the narrow band emission by LED’s. This implies that fluorescence as measured by the FIS carries information about the complete pool of photosynthetic pigments in the sample, not only chlorophyll. However, this makes the signal more complex to decipher.

Recording reference images prior to each experiment guarantees calibration of the system. In this way possible fluctuations of light intensity due to aging of the lamps are accounted for. In addition, light intensity is checked regularly and lamps are replaced when necessary. Maintenance of the liquid filters requires only regular filling up of the bottles to keep the level of liquid constant. If this is neglected, the level of the filter may decrease as low as to allow white light trough, which is readily detectable by an abnormal increase in brightness of the image.
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2.3 The Imaging Unit

2.3.1 Description

The imaging unit consists of a b/w CCD camera Sony XC-75 (Sony Corporation, Tokyo, Japan) equipped with a red cut-off filter B+W 092 (Schneider Optics Inc., Hauppauge, NY, USA), corresponding to Schott RG 695 mounted on the camera lens. Technical specifications of the camera are reported in table 2-1.

CCD cameras give, within a specific range, a linear response to light intensity. This means that the intensity of the video signal in a specific area of the image is linearly correlated to intensity of the light that reaches the sensor. In the specific case of fluorescence imaging the signal is then proportional to the intensity of the fluorescence emitted by the sample. This feature allows using the video signal delivered by the camera to perform quantitative measurements. However, in order to obtain numerical values for the light intensities, it is necessary to convert the analog signal from the camera into a digital one. The frame grabber interface card on the control unit, as described in section 2.4, performs this operation.

The automatic gain control of the camera is turned off in order to obtain a linear response of the CCD sensor to the fluorescence intensity in the range of interest. Figure 2-8 shows the response of the camera to various light intensities. The NTSC b/w video signal is then carried to the frame grabber in the control unit for digitization.

The red filter on the lens limits the light detection by the camera to the far-red/near-IR region of the electromagnetic spectrum, where the chlorophyll fluorescence is located. The red trace in figure 2-4 shows its transmission spectrum.

| Sony XC-75 |  
|---|---|
| **Pick up device** | 1/2" interline transfer (Hyper HAD sensor) |
| **Picture elements** | 768(H)x494(V) |
| **Cell size** | 8.4(H)x9.8(V) µm |
| **Lens mount** | C mount |
| **Sync system** | Internal/External (auto) |
| **H/V resolution** | 570x485 TV lines |
| **Sensitivity** | 400 Lux. F4(3200°K) |
| **Minimum sensitivity** | 3 Lux. F1.4 (with IR cut filter) |
| **S/N ratio** | 56dB |
| **Power** | DC 12V ± 10% / 1.6W |
| **Dimensions** | 44(W)x29(H)x71(D) mm |
| **Weight** | 140g |
| **Standard** | FCC, UL |

Table 2-1 Technical specifications of the camera Sony XC-75.
2.3.2 Technical Issues

Two main issues need to be addressed concerning the imaging unit: sensitivity of the camera and transmission spectrum of the filter used to detect fluorescence.

The CCD camera used in the system has a quite low sensitivity (3 lux) even when compared to low-priced consumer products on the market today. A system with a more sensitive camera (0.5 lux) is under development in our laboratory. The reason why we keep this configuration in the FIS is due to backward compatibility with data collected in the earlier times of the project.

In the current configuration, the FIS is equipped with a cut-off high-pass red filter. Such a set-up does not allow discriminating the two peaks that chlorophyll fluorescence exhibits in vivo at wavelengths around 690 and 735 nm. This is necessary, due to the low sensitivity of the Sony XC-75 camera. An interference filter would allow detecting only a very faint fluorescence signal, causing the signal/noise ratio to drop below acceptable limits. The alternate system currently under development in our laboratory overcomes this problem by using an interference filter and a more sensitive CCD camera.
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2.4 The Control Unit

2.4.1 Description

The control unit consists of a personal computer based on the Intel Pentium II processor (Dell Dimension XPS R450, Dell Computer Corporation, Round Rock TX, USA) equipped with a frame grabber (Osprey-100, Osprey Technologies, Inc., Morrisville NC, USA) and an AD/DA converter board (PCL-711B, Advantech Co., Ltd., Sunnyvale CA, USA). The operating system used is the UNIX-like system Linux, distribution Slackware 7.0, kernel version 2.0.13. This OS has been chosen having the advantage of the availability of a large body of freeware software, high flexibility in terms of software development and high reliability.

The system can deliver 8-bit gray-scale images with a maximal resolution of 640x480 pixels. Multiple frames (typically 3, but the number is user-selectable) are accumulated with the aim of reducing the noise in the picture.

The computer, by means of a custom-made interface (figure 2-9) connected to the digital output of the AD/DA converter board drives the illumination system by operating relay switches that turn on and off the lamps. The custom made interface is actually composed of an optocoupler and light emitting diodes (LED). The optocoupler isolates the two circuits, the lamp switch and the computer board, in order to protect the computer board from possible power spikes due to the relay-switch activity. The LED’s indicate the on/off status of each lamp.

The whole system is completely automated with the custom-made software package Grabix, developed by the author. Specifications and details of Grabix are reported in Appendix A. The software allows performing chlorophyll fluorescence imaging with several customizable protocols (see Appendix A). In a typical experiment, grayscale images with a
resolution of 640x480 pixels, which corresponds to an area of 8.5x6.4 cm, are captured at various time intervals: The first image is grabbed within 1 s from the onset of the illumination, and then images are grabbed at time intervals of 2, 5, 10, 20, 30, 60, 90, 120 and then every 60 s up to ten minutes. Experiments can be performed in continuous light or with pulses. In the latter case only the actinic light is continuously on during the experiment, and images are taken just before and then during saturating flashes fired at the time intervals described above.

### 2.4.2 Technical Issues

An alternative solution to the use of an electronic switch to drive the lamps could be the use of shutters. This latter solution presents, however, some difficulties concerning the engineering of the systems. The fiberoptic bundles used in the FIS are rather thick, requiring the use of a custom-made shutter. Such shutter, built in whatever way, would be relatively slow. Since the system has been designed to deal mostly with the slow phase of fluorescence emission, we opted for the cheaper and easier to implement solution: the electronic switch. We are aware that this solution implies a degree of inaccuracy due to the warm-up phase that the lamps require to reach full intensity. On the other hand, there are several advantages of this solution. Life of the lamp is preserved, as there is no need to keep it turned on all the time. Decline of lamp intensity due to excessive warming up during a day of work is limited. Maintenance of the switch is practically null, as there are no electro-mechanic parts involved.

The interfacing of the electronic switch with the main computer is done in the FIS with an AD/DA converter board. This solution is neat and convenient, but is not the only possible one. The standard parallel port present on the computer could be used for the same purpose, by connecting the switching device to the data pins of the port and by programming it accordingly.
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2.5 The Image Processing

The images as acquired by the system need to be digitally processed in order to give a quantitative interpretation of them. The required image processing routines vary from experiment to experiment depending on the protocol used, the material to be analyzed and the kind of information desired. There are, however, several common steps that need to be taken for any image acquired by the system. In this section I will describe only these common operations, leaving the description of the specialized ones to later sections.

2.5.1 Description

Most of the image processing is performed with the (free) student version of the (commercial) KhorosPro2001 suite (Khoral Research Inc., Albuquerque NM, USA). Details about this software suite are reported in Appendix B.1. The images delivered by the system are stored on the hard drive in the form of raw integer (32-bit) data files. The images in this form are imported into a format manageable by the software and then processed.

Figure 2-10 shows simplified schematics of the image-processing protocol used for chlorophyll fluorescence images. Two operations need to be performed on every image in order to prepare it for further analysis: dark signal subtraction and intensity correction. The first operation is needed in order to avoid an overestimate of the fluorescence signal. The second one is needed to correct for differences in the excitation light intensity across the camera field, as well as for possible differences in the transmittance at the edge of the camera lens. To perform these corrections two reference images are needed: one, called “dark”, representing the dark signal of the camera and a second one - “calibration” - which represents the intensity variations across the imaged field. The dark image is acquired by simply grabbing a video frame with the illumination lights off (i.e. darkness inside the cabinet). The calibration image is acquired by taking an image of a TLC plate, Silica Gel 60 (Merek & Co., Inc., Whitehouse Station, NJ, USA), dimensions 20 x 20 cm, placed on the object support. The TLC plate has been chosen as it provides a smooth and homogeneous white lambertian surface.
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What is an image histogram?
In an image histogram the distribution of the frequency of the intensity values is plotted on a chart. The chart on the left shows an example of such histogram. The frequency is calculated as the number of image pixels having that particular value. In our case, the number of classes for such histogram is 256, corresponding to the number of gray levels of the 8-bit image. Values on the x axis represent fluorescence intensity using pixel values as relative units. Values on the y axis represent the number of pixels in the image having a pixel value equal to x. Sometimes on the y axis a probability is reported instead, obtained by dividing the number of pixels by the total number of pixels of the image. The chart on the right side of the figure shows an alternative representation of the same distribution, in the form of integrated histogram. The information presented in this kind of chart is basically the same as in the normal frequency histogram, with the only difference being that the y values represent the probability of a pixel to have a value less or equal to x instead of simply equal to x.

The correction is done in three steps: first the dark image is subtracted from the sample image; then the result is divided by the background image (from which the dark signal has also been subtracted); finally the result is multiplied by a constant given by the mean intensity value of the background image for normalization purposes. Prior to these operations, the data type of the images needs to be converted from integer to floating point in order to make image division pixel by pixel. As the final step of the processing, images are saved in 8-bit ASCII format.

Multiple regions of interest (ROI’s), usually of 3x3 pixels corresponding in a typical setup to 0.16 mm², can be selected on each series of corrected images to follow the time course of the fluorescence quenching. The fluorescence value of each ROI is calculated as the integral of the pixel values in the ROI. The value is then normalized dividing by the difference of the value of the same ROI at time t=1 sec and time t=600 sec in order to normalize against local differences in chlorophyll content. The same procedure can be applied to wider areas or the whole image.
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In order to enhance the visualization, the histogram of the images can be clipped to eliminate background noise and stretched to improve contrast. When necessary, false colors can be applied.

*Applying false colors*

In order to enhance visibility and highlight fine details of the images, an artificial color map can be applied to fluorescence images. Several different maps among those available with the software package Khoros (see appendix B1) have been tested. The so-called *SA-pseudo*, named after the magazine *Scientific American* where it appeared first, has been selected. Figure 2-11 shows it next to a standard gray-scale. Blue/Black corresponds to low fluorescence values. Yellow/White corresponds to high fluorescence values. This particular scale has been chosen, as it preserves a natural transition from dark to light tones, yet the colors at the various levels of intensity give enough contrast to highlight small intensity variations, as exemplified in figure 2-12.

![Figure 2-11 The SA-pseudo color scale.](image)

![Figure 2-12 The SA-pseudo color scale. On the left, a gray-scale fluorescence image of an apple. On the right, the same image after applying the color map.](image)
2.5.2 Technical Issues

The choice of an ASCII format for the storage of the processed images is justified by the fact that such format is readily importable by most of the software for data analysis. In particular, it is very convenient for use with R (see appendix B1).

In terms of storage requirement, a single raw image needs 1.17 MB of free disk space. Table 2-2 reports the space needed for typical experiments. Dark and calibration image (1.17 MB each) are not included in the count. For details about the protocols see appendix A.

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</tbody>
</table>

Table 2-2 Storage requirements for typical FIS experiments. Values are expressen in MegaBytes (MB)

For permanent storage the images are recorded on a compact disc (CD), which allows storing up to 650 MB of data. Alternatively, magnetic tapes can be used, which have a higher storage capacity (20 GB).
Chapter 3
Fluorescence Image Analysis of Apple Fruits

3.1 Introduction

Though the chlorophyll fluorescence imaging system described in the present thesis is open to a wide variety of applications, one of the most intriguing ones is in the domain of fruit quality assessment. Extensive investigation of apple fruit samples has been conducted by means of chlorophyll fluorescence imaging in order to test the suitability of such a technique as an additional non-destructive tool for the quality control of fruit.

In this chapter I will first describe the experimental steps required to perform the fluorescence image analysis of an apple fruit, some of which are specific to apple images. To clarify the experimental approach, a detailed description of the kinetics of fluorescence emission by an apple fruit will be given, as measured by the fluorescence imaging system, together with an overview of the image processing techniques used.

In the Results section, data from a particular experiment will be presented, which demonstrate how the chlorophyll fluorescence imaging system can be successfully applied for predicting the shelf life and estimate maturity of apple fruits. Then, data will be shown concerning image analysis of apple fruits coming from different geographical areas (Belgium and Spain) and treated with different water or nitrogen supply. The progress made in applying advanced analysis techniques will also be dealt with. These techniques include Artificial Intelligence methods such as neural network simulations. With neural networks it is possible to train an expert system (in our case a computer program) to recognize specific features of the images, in such a way that the system can then make decisions (such as sorting the samples) autonomously, based on the input data received from the imaging system. It will be shown that, though the results of this kind of analysis applied to apple images are not yet extremely satisfactory, they are nevertheless very encouraging.
3.2 Materials and Methods

3.2.1 Plant Material

The material for the experiments in section 3.3.1 and 3.3.2 consisted of Golden Delicious apples bought at a local store. For the maturity estimate experiment (3.3.2), the apples were kept on the shelf at room temperature and room light (fluorescent lamps) for 18 days. Measurement where conducted on day 0, 1, 3, 6, 8, 13, 15 and 17.

Apples for the experiments in section 3.3.4 and 3.3.5 where provided by the Royal Research Station of Gorsem, Belgium. Trees of the Jonagold varieties (*Malus x Domestica* Borkh. ‘Jonagold 2361’) at the test fields located in Velm were subjected to no Nitrogen fertilizer application (*N*) or to an extra supply of 30 Kg / Hectare (*N2*). In addition some parcels were subjected to Calcium-treatment (*CI* = no extra N and no Ca; *C3* = 9 times Ca-treatment during the fruit maturation period). Both latter localities are situated in the Haspengouw area of the province of Limburg: one of the main fruit producing regions of Belgium.

For the experiments in section 3.3.6 the apples where provided by the station for experimental agriculture Fundació Mas Badia, La Tallada, Catalonia, Spain. Apple trees (*Malus x Domestica* Borkh. ‘Golden Smoothee’) where grown in randomized experimental units composed of 3 rows of 9 trees each. All the trees in the orchard were clones of the same apple cultivar, which guarantees little genetic variability. To avoid interferences from the neighboring units, samples where collected only from two central trees of the central row. The experimental units where treated with several different irrigation regimes. The apples used in our experiments came from units treated with four different regimes:

\[ T1 = -10 \text{ KPa} \]
\[ T2 = -30 \text{ KPa} \]
\[ T3 = -60 \text{ KPa} \]
\[ T4 = -90 \text{ KPa} \]

Replicates of each treatment where collected from 4 different experimental units with the same treatment. The apple batches have been named according to the following convention: *TnRm*, with *n* = treatment and *m* = replicate. For example T2R4 = treatment nr. 2 replicate nr. 4.
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

Each year samples were harvested in October and measured in October, February and June. The data presented here refer only to the October measurements. More specifically Jonagold apples were harvested at two dates: first harvest (J1) between 8 and 15 September and the second one (J2) between 1 and 8 October 1999.

### 3.2.2 Imaging protocol for apple fruits

The common image processing steps required for any image taken by the system are described in section 2.5. For the analysis of chlorophyll fluorescence images of apple fruits, additional specific operations are required, namely the cropping and masking of the image. These operations, like the entire image processing procedures described in the present thesis, were performed with the help of the Khoros software suite (see Appendix B.1). Figure 3-1 shows an extended version of the general flow chart for the fluorescence image processing protocol (fig. 2-10), specifically adapted to apple fruit images. It includes the two additional steps of cropping and masking the image. These two image-processing operations are required in order to limit at least partially the problem caused by the fact that apple fruits have an irregular shape, which is neither spherical nor flat. If the shape of the fruit was spherical, a geometrical correction factor could be introduced.

**Figure 3-1 The image-processing protocol for apple fruits.** The steps common to all fluorescence images are shown in the green boxes. Two extra steps, image cropping and image masking, are represented by yellow boxes.
(see below). Attempts have been made in this direction, but the results were not satisfactory and the procedure has been discarded for several reasons.

![Diagram](image)

**Figure 3-2** Schematics of an ideally spherical apple. See text for explanations.

*The $\cos^2$ correction*

Based on the analysis of the geometry of an apple presented below, a geometrical correction factor represented by the squared cosine of the incidence angle of the excitation light can be introduced. Fig 3-2 shows schematically the cross-section of an ideally spherical apple. Let $I$ be the light intensity at the apple surface, $d$ the distance of a point from the center of the image (care must be taken to place the sample centered in the imaging field of the camera) and $\alpha$ the angle formed by the ray of excitation light and the line joining the point $P$ and the center $C$ of the apple. The angle $\alpha$ can be estimated as $\alpha = \arcsin(d/r)$ where $r$ is the radius of the "spherical" apple. The intensity of the light that actually hits the surface of the
Figure 3-3 Left: Chlorophyll fluorescence image of Golden Delicious apple. Right: the same image after the squared-cosine correction. The circular shape is due to the assumption that the apple is spherical. The color scale is as described in chapter 2. The contrast of the right image is modified as compared to the left one in order to enhance visibility.

Figure 3-4 Intensity profile of the images in figure 3-3. The profile refers to the horizontal line number 240 (i.e., the central line).

apple perpendicularly can then be estimated from $I \cos(\alpha)$. It follows that the fluorescence emission at that specific point is $F_{\text{point}} = k I \cos(\alpha)$ where $k$ is a constant. The fluorescence component that goes straight back to the camera is $F = F_{\text{point}} \cos(\alpha)$. By simple substitution we obtain $F = k I \cos^2(\alpha)$. As a result all the pixels of the image should be corrected introducing
the factor $\cos^2(\alpha)$, where $\alpha$ needs to be calculated for each pixel. Several assumptions are made in order to apply such a correction:

1) The apple is spherical.
2) The light intensity that hits the apple surface is the same at each point.
3) There is no light scattering at the apple surface, or it is negligible.

Obviously, all these assumptions make this model very weak. Apple fruits are indeed not quite spherical, often are far from being spherical and, though such model is probably the best we can apply, the increased computational complexity introduced doesn't pay off in terms of increased reliability of the image. Secondly, a correction factor would have to be introduced for the different intensities with which excitation light reaches surface points laying at different distances from the light source (increasing from the center of the image toward the periphery). Such a correction factor would be difficult to determine with sufficient accuracy, being dependent not only on the distance but also on the refractive index of the air, and hence on environmental factors like air humidity and temperature. Furthermore, the light beams originating from the fibers are not parallel, as a consequence of the optical properties of the fibers (the light bundle spreads out at an angle from the fiber termination) and their spatial arrangement in the construction, which introduces one more distortion factor.

Figure 3-3 shows an example in which the kind of correction just described has been applied. On the left is the image before correction and on the right is the image after correction. Figure 3-4 shows cross-section intensity profiles from the same two images. Clearly, the introduction of the squared-cosine correction factor does not flatten the profiles, indicating that this kind of correction is not suitable for the purpose. Furthermore, even if the correction was reliable from a geometric point of view, as a matter of fact the points laying at larger distances from the center are excited with much less light, due to the higher angle of incidence of the rays, which influences the behavior of those points in terms of kinetics of fluorescence emission. It can be expected that at the time when the image is grabbed, points excited by different light intensities are in a different state of the fluorescence emission.

All in all, the imprecision carried by the assumptions made when applying the spherical model make such a model unsuitable for the purpose of correcting the images. Furthermore, performing such correction implies an increased computational complexity,
which decreases the time performance of the system, slowing down considerably a possibly automated decision making process. This is unacceptable in the view of the intended application of the system to fruit grading lines, where time performance is critical.

Based on all the constraints described above, the squared-cosine correction has been rejected. Instead, a simpler solution has been adopted in order to minimize the error caused by the geometry of the fruit.

*Masking and cropping*

The solution mentioned in the previous section consists of the application of a circular mask to the picture, which limits the analysis to a round area with 3.2 cm diameter, represented by an image of 256x256 pixels. Of the 65536 pixels of the image only 51431 are actually used, the others belonging to the mask. Within the circular area extracted from the original image, the intensity variations of the excitation light are small. The intensity variations across the (flat) field of the camera are reduced to about 15% (red traces in figure 2-7) and the differences due to the curvature of the apple surface can be neglected. In fact, within the limits of the imaged area, the apple surface can be considered nearly flat. Figure 3-5 shows the three-step procedure adopted to prepare apple images for further analysis: the first two steps – dark signal subtraction and illumination intensity correction – have already been described in section 2.5; in the third step, beside cropping (not shown) and masking the image, the color depth of the image is reduced to 8-bit, i.e. 256 gray levels. The image is then exported to ASCII format in order to make the data available for importing by other software for further statistical analysis. False colors are applied and, when needed, contrast

*Figure 3-5* Processing of an apple fruit chlorophyll fluorescence image. Cropping of the image has been omitted for clarity. Intensity correction includes normalization to the mean intensity of the image.
enhancement is performed on the picture with the sole purpose of enhancing the visual quality of the images. Numerical analysis, however, is always performed on the image before enhancement.

There is another reason why it is necessary to crop and mask the apple image during its processing. A good quality image is a prerequisite in order to obtain reliable data. Even after correcting for the dark signal of the camera and the uneven distribution of the excitation light, the quality of the image as delivered by the instrument needs improvement, as proven by the histogram analysis presented in figure 3-6.

![Histograms](image)

**Figure 3-6** Normal (top) and integrated (bottom) histograms of a typical apple fluorescence image before (left) and after (right) masking.

The two charts on the left belong to an apple image before processing, the two on the right to the same image after processing. The normal histogram before processing (fig 3-6 top-left) shows strong skewness towards low values, with a large peak for pixel values close to 0. The pixels in the tail represent points belonging to the edge of the apple, with low intensity fluorescence data. The peak around x=0 is formed by the background of the image. If we used the image as such, those points would bias heavily the statistical analysis (see next section) of the image. Applying the image-processing protocol described above results in an image of much better quality, as shown by the right side histograms of figure 3-6. The normal
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

histogram after masking and cropping (fig. 3-6 top –right) shows a typically bell-shaped distribution of the data values.

Even more evident is the effect in the integrated histogram, which shows a clear S-shaped curve. Having a distinct, reliable integrated histogram plot is very important for comparing images, be it of the same sample at different times or of different samples.

3.2.3 Statistical analysis and parameterization of apple fruit images

The images obtained by the processing technique described in the previous section are subsequently statistically analyzed. All the statistical analyses are performed with the “R” statistical package (see Appendix B2).

Figure 3-7 Histogram plot of the distribution of the intensity values of the fluorescence image the apple shown in figure 3-5. The vertical lines represent, from left to right, the 1st, 5th, 25th, 50th, 75th, 95th and 99th percentile.
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

Each image can be considered to be a set of 65536 point measurements of fluorescence intensity, of which actually only 51431 are used; the other 14105 belong to the mask and have a value equal to 0. When analyzing the image histogram, it becomes immediately evident that it presents an asymmetric shape. This is a consequence of the fact that the fluorescence values are not normally distributed. Lazar and Nauš (1998), in a study of the statistical properties of chlorophyll fluorescence parameters, have shown that for relatively large samples the distribution of the values of several parameters from the fast fluorescence induction curve (Strasser et al., 1995) measured with the Plant Efficiency Analyser (Hansatech, UK) was not normal. They suggested to better use median, quartiles, minimum and maximum to present fluorescence data, instead of mean and standard deviation, which is what we will do here.

For comparison purposes and in order to prepare vectors for neural network analysis (see the following section), the images need to be parameterized, i.e. a set of numerical parameters has to be calculated based on the features of the image. We parameterize the images on the basis of the distribution of the pixel values of the image. For each image ten such parameters are calculated:

- 1st percentile
- 5th percentile
- 25th percentile
- 50th percentile (Median)
- 75th percentile
- 95th percentile
- 99th percentile
- Mean
- Standard deviation
- Slope
- Intercept
- \( R^2 \)

Instead of the minimum and the maximum, the 1st and 99th percentile of the distribution of the pixel values were calculated. This was necessary in order to avoid artifacts due to the signal noise of the camera, resulting in dark or bright spikes. The percentiles give a better estimate of what the minimum and the maximum of the distribution look like. Yet, for very skewed distributions like the one in figure 3-7, the estimate of minimum and maximum

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could be somewhat inaccurate. For our purpose the selected percentile values provide a
practical parameterization of the distribution of the intensity values. Figure 3-7 shows the
histogram of a fluorescence image of a typical apple (fig. 3-5). The vertical lines represent the
percentiles as described above. All these parameters are expected to vary in case of stress or
in case of altered physiological conditions. The symmetry of the distribution is also expected
to change in case of stress.

Mean and standard
deviation of the distribution are
calculated in order to obtain
values to be used to simulate a
normal distribution with the same
mean and standard deviation.
Such a simulated distribution is
then used to carry out the
following normality test. The
simulated normal distribution and
the real distribution are plotted
against each other - after sorting
of the values - in a scatter plot to
give a result like in figure 3-8.
The linear regression is then
calculated and the slope and
intercept of the regression line are

extracted. The correlation coefficient $R^2$ is also computed.

The first seven parameters where chosen because they carry information about the
average intensity of fluorescence emission, the minimum, the maximum and how much the
intensity values are spread around the average.

With the normality test we expected to have an indication of how far from normality
the distribution is, which is an indication for the physiological conditions of the sample. Data
in support of this view are presented in section 3.3.3.
3.2.4 Artificial Neural Network (ANN) Analysis

An artificial neural network is an information-processing system modeled after natural neural networks like the human brain. It consists of a large number of simple processing elements called neurons, units, cells or nodes (Fausett, 1994). These elements are interconnected by communication links, each with an associated weight (fig 3-9). Artificial neural networks can be implemented in various ways. The most common is by means of computer programs. In our case we used the Stuttgart Neural Network Simulator version 4.1 from the Institute for Parallel and Distributed High Performance Systems of the University of Stuttgart, Germany. Details about this software package are given in appendix B3.

![Neural Network Diagram](image)

**Figure 3-9** Backpropagation neural network with one hidden layer (Z). X = input layer. Y = output layer. From Fausett (1994).

Neural networks are capable of solving a variety of problems like, among others, pattern recognition and pattern classification. In the present work, we use ANN’s to perform pattern classification on fluorescence images of apples. By pattern we don’t mean visual patterns visible on the image itself, for using the whole image for such analysis would be computationally enormously demanding. Rather we use vectors with dimension n=12 extracted from the images as described in the previous section, or with dimension n=24 created by merging the two vectors for the green and the red side of the apple.
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

When provided with an input vector, the units of a neural network will fire in a
cascade signals to the units in the next layer, according to a specific activation function and to
the weights of the connections, down to the output layer. As a result, the output units will
obtain each a value, which together form the output vector. To make an artificial neural
network capable to recognize patterns means that the ANN must be trained so that once fed
with an unknown input vector, it is able to produce a specific output vector indicating to what
class the input vector belonged. For example, in the case of the apple images the ANN must
be able to recognize to which class of, say irrigation regime the apple belongs. In order to
train the network we need a training set and a test set. The training set consists of a series of
couples of vectors (input and output) one for each apple. The test set consists only of input
units. During the training phase the ANN is fed for a number of cycles with the training set.
At each cycle the network calculates an error function indicating how far the network was
from the correct answer, and adjusts the weights in order to minimize this function. When the
error function has reached a minimum, the network is fully trained. At that moment, the
network can be tested with the test set. In other words, unknown vectors are fed to the ANN
and the resulting output vectors are checked for correctness of classification. The training
phase is usually time consuming. Once the network is fully trained, the classification phase is
very fast.

In the present work we use a standard back-propagation network made of 12 or 24
input units (see above), 16 hidden units and 2 or 4 output units, depending on the experiment.
The number of training cycles varies with the experiment, always being in the order of tens of
thousand. The number of cycles is chosen by following the evolution of the error function
until it reaches a stable minimum. The recognition rate is calculated as the percentage of
patterns classified successfully. This value is always reported for both the test set and the
whole (test + training) set.
3.3 Results

3.3.1 Fluorescence induction kinetics (Kautsky effect) of an apple fruit as measured by the chlorophyll fluorescence imaging system

As pointed out in section 2.4, the imaging system is capable of grabbing fluorescence images with several customizable protocols. In this section I will give a description of the kinetics of chlorophyll fluorescence emission by an apple fruit, as measured by the imaging system on a time scale of several minutes. To this purpose, a Golden Delicious apple was used (fig. 3-10). The sample was not dark-adapted, but room-light-adapted. This choice was made basically because of two reasons: firstly, in view of an in-line application of the system on grading machines, where a long dark adaptation would pose a non-trivial technical problem, room-light-adaptation would be the preferred condition; secondly, room the light intensity is very low as compared to the one used by the instrument (5 vs. 1100 μE m$^{-2}$ s$^{-1}$), and a sample adapted to this conditions has an induction kinetics of fluorescence emission comparable to the one obtained with a fully dark adapted sample. The system was programmed to shine intense light (1100 μmol s$^{-1}$ m$^{-2}$, λ<650 nm) on the sample and grab images at the following time intervals (in seconds from the onset of the illumination): 1, 2, 5, 10, 20, 30, 60, 120, 180, 240 and 300.

Figure 3-11 shows the sequence (left to right, top to bottom) of images from one sample. The color scale is reported on the left side of the image set. The fluorescence quenching kinetics develops relatively homogeneously over the whole imaged area. A ring-shaped area of high fluorescence emission is visible around the lenticels, which is not observable by eye.

Figure 3-10 Photograph of the Golden Delicious apple used in the experiment described in section 3.3.1. The red circle corresponds to the area shown in the fluorescence images after applying the circular mask.
Figure 3-11 Kinetics of fluorescence induction of a Golden Delicious apple as measured by the fluorescence imaging system described in chapter 2. From left to right, top to bottom, fluorescence images after 1, 2, 5, 10, 20, 30, 60, 90, 120, 180, 240 and 300 seconds under continuous excitation.
When we extract from the images the pixel intensity values as a measure of the fluorescence intensity and we plot the median of those values, we obtain a chart like in figure 3-12. The curve on the chart looks like, and in fact is, a typical Kautsky-curve (quenching phase). Advantage of having a digital imaging system is that we can restrict the analysis to small regions of interest within the image. In figure 3-13 is reported an example in which the fluorescence induction curve has been extracted from the images for three points: one corresponding to a lenticel, one corresponding to the area surrounding the lenticel (Aureola) and one corresponding to a point between the lenticels. Fluorescence values are calculated as median of the values over an area 3x3 pixel wide. If we analyze the raw fluorescence curve (fig. 3-13 top), the result is what we would expect from the visual inspection of the picture: lenticels emit low fluorescence, aureola’s emit a stronger signal and other points have values somewhere in between. More information is obtained after normalizing the fluorescence values as $F_{\text{norm}} = (F_1 - F_{300})/(F_1 - F_{300})$, where $F_{300}$ is the fluorescence at the steady state (300 sec). This kind of normalization allows analyzing the kinetics of fluorescence emission.
independently from absolute intensity values, which may differ for several reasons. It
becomes evident (fig 3-13 bottom) that the kinetics of fluorescence quenching doesn’t differ
remarkably between the three different points. This is what we could expect from a healthy
apple like the one of this example, which doesn’t carry any superficial defects.
Figure 3-13 Slow phase of the fluorescence induction kinetics of three different points of the peel of an apple, as extracted from the images in figure 3.11. *Aureola* = ring-shaped area surrounding the lenticel. *Other* = area between the lenticels. Top: raw data chart. Bottom: data normalized as $(F_t-F_{300})/(F_t-F_{300})$. 

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3.3.2 Chlorophyll fluorescence imaging as a tool to estimate apple maturity / shelf life

Fluorescence emission from the peel of apple fruits has been reported before to decline with storage time of the apples (Song et al. 1997). The idea behind the experiment presented in this section is exploiting this feature to use the chlorophyll fluorescence imaging system as a tool to assess in a fast, visual way the physiological age of an apple.

In order to investigate such possibility, a batch of Golden Delicious apples was kept for several days at room temperature and the fluorescence emission monitored. Figure 3-14 shows (from left to right, top to bottom) photographs of one sample from the batch after 0, 1, 3, 6, 8, 13, 15 and 17 days after purchasing it at a local store. The only visible change during this time was a slight shift of the yellow hue from greenish to more yellowish. No other analysis than fluorescence imaging was performed on the apples, as the goal was to follow the changes for each and every apple. Destructive analyses, hence, where not feasible.

![Figure 3-14 Photographs of a Golden Delicious apple during storage at room temperature. Left to right, top to bottom: the same apple after 0, 1, 3, 6, 8, 13, 15 and 17 days after purchasing it at a local store.](image)

Figure 3-15 shows fluorescence images of the same apple. Images where taken during a saturating light pulse on the room-light adapted apple, and represent $F_M$.

The fluorescence decline over time is fairly homogeneous over the whole imaged area, though the areas around the lenticels emit a more intense signal. After the third day of shelf storage, the decline in fluorescence emission is evident. This becomes more conspicuous
if we plot the integrated histogram for each image (fig. 3-16).

**Figure 3-15** Chlorophyll fluorescence images of a Golden Delicious apple during storage at room temperature. Left to right, top to bottom: the same apple after 0, 1, 3, 6, 8, 13, 15 and 17 days after purchasing it at a local store. The color scale is reported on the left side of each image.

**Figure 3-16** Integrated histogram from the chlorophyll fluorescence images of the Golden Delicious apple in figure 3-15
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

The decline in fluorescence emission results in a shift of the sigmoid curves toward low pixel values, which correspond to low fluorescence intensity. After the third day the values flatten out on the low side of the scale, as becomes clear from the plot of the average fluorescence intensity of the images, calculated as median of the pixel values over the whole image, versus time (fig. 3.17). Within a week the fluorescence emission reaches a plateau and declines further after two weeks.

**Figure 3-17** Average fluorescence intensity from the images in figure 3-15 versus shelf storage time. The average is calculated as median of the pixel values of the image.
3.3.4 An example in which chlorophyll fluorescence imaging anticipates the occurrence of a disorder

During the experiment presented in the previous section, some of the samples developed some sort of disorder at various stages during the three weeks of room storage. For some of them the occurrence of it could be predicted by analysis of the fluorescence image. Figure 3-18 and 3-19 show color and fluorescence images of a “good” (3-18) and a “bad” (3-19) apple before (day 3) and after (day 8) the development of scald in one of them. At day three neither apple showed any visual symptoms that could allow anticipating the possible occurrence of a storage disorder. The fluorescence images were fairly homogeneous in both cases, but the “bad” apple presented already a higher fluorescence emission as indicated by the higher average fluorescence for this apple (table 3-1). A careful analysis of the distribution of the pixel values (figure 3-20) has evidenced a fairly normal distribution in the case of the “good” apple, and a less regular distribution for the “bad” one. When the normality test

Figure 3-18 Top left: a Golden Delicious apple. Top right: the same apple after five days of room temperature storage. Bottom left: fluorescence picture from the area indicated by the red circle. Chart: normality test for the fluorescence picture (normal vs. actual distribution).
described in section 3.2.3 was applied (charts in figure 3-18 and 3-19, results in table 3-1), a lower value for $R^2$ was found for the “bad” apple as compared to the “good” one (0.980 vs. 0.991), indicating a “less normal” distribution of the fluorescence values over the area already before the actual development of the scald.

<table>
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<tr>
<td>$R^2$</td>
<td>0.991</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Table 3-1 Parameters extracted from the fluorescence pictures of figure 3-18 and 3-19, calculated according to the procedure described in section 3.2.3
Figure 3-20 Histograms of the distributions of the intensity values for the fluorescence images in figure 3-18 and 3-19.
3.3.5 Effect of Nitrogen fertilization on fluorescence emission by apple fruit cv Jonagold.

Figures 3-21 and 3-22 and table 3-2 show the results from the statistical analysis of the whole set of images from the green side of the 160 apples of the nitrogen fertilization experiment. Samples were taken at two different times of harvest, as indicated by the number associated with the letter J. Control samples are marked as N1; samples from the orchard fertilized with nitrogen are marked as N2. The data presented refer to the parameter extracted from the images according to the image processing protocol described in section 3.2. Values are reported as median of the 40 samples for each plot. In the upper part of the table the data from the red side of the apples are reported, while in the lower part those from the green side are presented. Figure 3-21 shows on a chart the seven percentile values selected from the image histogram, averaged (as median) over the two harvests (J1 and J2). Figure 3-22 presents the data split with respect to harvest date. The
higher values observed in the nitrogen treated samples (green side) reflect a shift of the image histogram toward high pixel values, i.e. an overall increase in fluorescence intensity over the imaged area.

This is true at both harvests, though more pronounced at the earliest (J1). Fluorescence values from the red side of the apple don't differ substantially in the treated and non-treated samples. The remaining data extracted from the images, i.e. mean, standard deviation, intercept, slope and R² from the normality test, are reported in table 3-2. Mean and standard deviation values, as expected, confirm the changes observed by analyzing the various percentiles from the image histogram. Intercept and slope values are also reported, though the physiological meaning of them is not obvious. Correlation values (R²) indicate an increased correlation, i.e. a "more normal" distribution, associated with the increase in fluorescence emission from the green side of the nitrogen-treated apples.

Artificial neural network analysis (table 3.3) was only partly successful. When vectors from only the green or the red side image of the apples were used for training, the network was
not able to learn. In other words, the error function (not shown), though slowly decreasing, didn’t converge to low (close to 0) values.

When the vector of size \( n=24 \) resulting from the merger of the vectors for the two sides of the apple was used for the training, the system was able to recognize quite accurately the training set (93\% of success), and with a lower rate also the (unknown) test set (65\%). An overall recognition rate of 79\% was achieved.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Intercept</th>
<th>Slope</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1N1</td>
<td>49</td>
<td>6.10</td>
<td>0.696</td>
<td>0.987</td>
<td>0.975</td>
</tr>
<tr>
<td>J1N2</td>
<td>38</td>
<td>5.35</td>
<td>0.373</td>
<td>0.987</td>
<td>0.975</td>
</tr>
<tr>
<td>J2N1</td>
<td>29</td>
<td>5.89</td>
<td>0.398</td>
<td>0.987</td>
<td>0.973</td>
</tr>
<tr>
<td>J2N2</td>
<td>32</td>
<td>4.42</td>
<td>0.418</td>
<td>0.987</td>
<td>0.973</td>
</tr>
<tr>
<td><strong>Green side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1N1</td>
<td>87</td>
<td>10.04</td>
<td>0.887</td>
<td>0.988</td>
<td>0.976</td>
</tr>
<tr>
<td>J1N2</td>
<td>115</td>
<td>11.84</td>
<td>0.907</td>
<td>0.990</td>
<td>0.979</td>
</tr>
<tr>
<td>J2N1</td>
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<td>10.56</td>
<td>1.050</td>
<td>0.985</td>
<td>0.969</td>
</tr>
<tr>
<td>J2N2</td>
<td>98</td>
<td>10.08</td>
<td>0.882</td>
<td>0.991</td>
<td>0.981</td>
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<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Intercept</th>
<th>Slope</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JN1</td>
<td>39</td>
<td>6.00</td>
<td>0.547</td>
<td>0.987</td>
<td>0.974</td>
</tr>
<tr>
<td>JN2</td>
<td>35</td>
<td>4.89</td>
<td>0.396</td>
<td>0.987</td>
<td>0.974</td>
</tr>
<tr>
<td><strong>Green side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JN1</td>
<td>84</td>
<td>10.30</td>
<td>0.969</td>
<td>0.986</td>
<td>0.973</td>
</tr>
<tr>
<td>JN2</td>
<td>107</td>
<td>10.96</td>
<td>0.895</td>
<td>0.990</td>
<td>0.980</td>
</tr>
</tbody>
</table>

Table 3-2 Values extracted from the histogram of fluorescence images of Jonagold apples. J* N*: values split with respect to harvest date (J1 = first harvest, J2 = second harvest) and treatment (N1 = control, N2 = extra nitrogen supply). JN*: value grouped by harvest time. SD = standard deviation.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Training</th>
<th>Test</th>
<th>Training + Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red side alone</strong></td>
<td>Learning unsuccessful</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Green side alone</strong></td>
<td>Learning unsuccessful</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Green + red</strong></td>
<td>93%</td>
<td>65%</td>
<td>79%</td>
</tr>
</tbody>
</table>

Table 3-3 Artificial neural network analysis of fluorescence images of Jonagold apples. Recognition rate is reported as percentage of data vectors correctly classified by the system.
3.3.6 Effect of Calcium treatment on apple fruit cv Jonagold.

A set of measurement and analyses analogous to the one described in the previous section was performed on Jonagold apples treated or not with extra Calcium sprays, a common practice used to harden cell walls and improve post-harvest storage potential of the fruits. The percentile values reported in figure 3-23 indicate that both the red and the green side of the fruit showed an increased fluorescence emission in the Ca-treated samples. Such effect was more pronounced at the first harvest for the green side of the apples, and more at the second harvest for the red side of the fruit (figure 3-24). No remarkable differences were observed concerning the shape of the distribution of the fluorescence values, as evidenced by the similar $R^2$ values found by the normality test (table 3-4).

Neural network analysis (table 3-5) was successful when using vectors extracted from the green side images, but also with the combined vectors from the two images from the
green and the red side. Learning by the ANN was unsuccessful when the vector from the red side image alone was used.

Figure 3-24 Percentile values extracted from the histogram of fluorescence images of Jonagold apples. Values split with respect to harvest date and treatment. Top: red side of the apple. Bottom: green side. C1 = no Ca treatment. C3 = sprayed with Ca (see text).
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Intercept</th>
<th>Slope</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1C1</td>
<td>45</td>
<td>4.95</td>
<td>0.452</td>
<td>0.990</td>
<td>0.979</td>
</tr>
<tr>
<td>J1C3</td>
<td>49</td>
<td>6.66</td>
<td>0.394</td>
<td>0.989</td>
<td>0.979</td>
</tr>
<tr>
<td>J2C1</td>
<td>34</td>
<td>5.57</td>
<td>0.586</td>
<td>0.984</td>
<td>0.968</td>
</tr>
<tr>
<td>J2C3</td>
<td>42</td>
<td>7.24</td>
<td>0.461</td>
<td>0.987</td>
<td>0.974</td>
</tr>
<tr>
<td><strong>Green side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J1C1</td>
<td>101</td>
<td>12.22</td>
<td>1.064</td>
<td>0.988</td>
<td>0.977</td>
</tr>
<tr>
<td>J1C3</td>
<td>134</td>
<td>13.57</td>
<td>1.959</td>
<td>0.984</td>
<td>0.968</td>
</tr>
<tr>
<td>J2C1</td>
<td>98</td>
<td>10.28</td>
<td>0.766</td>
<td>0.989</td>
<td>0.978</td>
</tr>
<tr>
<td>J2C3</td>
<td>106</td>
<td>11.21</td>
<td>0.856</td>
<td>0.990</td>
<td>0.979</td>
</tr>
</tbody>
</table>

|               |      |     |           |       |     |
| **Red side**  |      |     |           |       |     |
| JC1           | 40   | 5.26| 0.519     | 0.987 | 0.974 |
| JC3           | 46   | 6.95| 0.428     | 0.988 | 0.976 |
| **Green side**|      |     |           |       |     |
| JC1           | 99   | 11.25| 0.915   | 0.989 | 0.978 |
| JC3           | 120  | 12.39| 1.407  | 0.987 | 0.973 |

Table 3-4 Values extracted from the histogram of fluorescence images of Jonagold apples. J*C*: values split with respect to harvest date (J1 = first harvest, J2 = second harvest) and treatment (J1 = control, J3 = Ca-treated). JC*: value grouped by harvest time. SD = standard deviation.

<table>
<thead>
<tr>
<th>Data set</th>
<th>Training</th>
<th>Test</th>
<th>Training + Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red side alone</td>
<td></td>
<td></td>
<td>Learning unsuccesful</td>
</tr>
<tr>
<td>Green side alone</td>
<td>99%</td>
<td>60%</td>
<td>80%</td>
</tr>
<tr>
<td>Green + red</td>
<td>98%</td>
<td>66%</td>
<td>82%</td>
</tr>
</tbody>
</table>

Table 3-5 Artificial neural network analysis of fluorescence images of Jonagold apples. Recognition rate is reported as percentage of data vectors correctly classified by the system.
Chapter 3 - Fluorescence Image Analysis of Apple Fruits

3.3.7 Effect of irrigation regime on apple fruits cv Golden Smoothee

The effect of four different irrigation regimes was investigated on apple fruits cv Golden Smoothee by means of chlorophyll fluorescence imaging.

Figure 3-25 shows the percentile values extracted from the histogram of the fluorescence images from the green side of the apples, according to the procedure described in section 3.2.3. Figure 3-26 shows the same for images from the opposite (yellow) side of the apples. Figure 3-27 shows the average of the two sides. It is evident that the differential irrigation regime did not have any influence on the fluorescence emission as measured by the fluorescence imaging system, though there is some difference between the two sides of the apples, the greener showing a higher fluorescence signal. Results from the normality test (table 3.6) also show no particular differences due to the different water treatment. Neural network analysis (data not shown) has been attempted, but with limited success.
Chapter 3 – Fluorescence Image Analysis of Apple Fruits

![Graph showing fluorescence values across percentiles for different stress levels](image)

**Figure 3-27** Percentile values extracted from the histogram of fluorescence images of Golden Smoothie apples. Average of both sides of the apple. $T_1 = -10 \text{ KPa}$, $T_2 = -30 \text{ KPa}$, $T_3 = -60 \text{ KPa}$, $T_4 = -90 \text{ KPa}$.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>Intercept</th>
<th>Slope</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Green side</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_1$</td>
<td>82.4</td>
<td>7.62</td>
<td>1.12</td>
<td>0.986</td>
<td>0.971</td>
</tr>
<tr>
<td>$T_2$</td>
<td>85.1</td>
<td>8.67</td>
<td>1.17</td>
<td>0.986</td>
<td>0.971</td>
</tr>
<tr>
<td>$T_3$</td>
<td>79.4</td>
<td>8.58</td>
<td>1.31</td>
<td>0.982</td>
<td>0.965</td>
</tr>
<tr>
<td>$T_4$</td>
<td>82.6</td>
<td>10.1</td>
<td>1.27</td>
<td>0.985</td>
<td>0.969</td>
</tr>
</tbody>
</table>

| **Yellow side** |      |     |           |       |       |
| $T_1$     | 63.0 | 7.89| 0.996     | 0.983 | 0.966 |
| $T_2$     | 67.8 | 8.08| 0.976     | 0.983 | 0.967 |
| $T_3$     | 62.6 | 9.11| 0.919     | 0.984 | 0.969 |
| $T_4$     | 65.1 | 9.76| 1.12      | 0.982 | 0.964 |

| **Average** |      |     |           |       |       |
| $T_1$     | 72.7 | 7.75| 1.06      | 0.984 | 0.969 |
| $T_2$     | 76.5 | 8.38| 1.07      | 0.984 | 0.969 |
| $T_3$     | 71.0 | 8.84| 1.11      | 0.983 | 0.967 |

**Table 3-6** Values extracted from the histogram of fluorescence images of Golden Smoothie apples. $T_1 = -10 \text{ KPa}$, $T_2 = -30 \text{ KPa}$, $T_3 = -60 \text{ KPa}$, $T_4 = -90 \text{ KPa}$. SD = standard deviation.
3.4 Discussion

Analyzing fluorescence images of apples in various contexts revealed the promising potential of the technique as an additional tool to assess fruit quality. Chlorophyll fluorescence is a signal rich in information, but still challenging to decipher. Measuring it in two dimensions increases even more its complexity. From a technical standpoint, the fluorescence imaging system presents two major limitations: low time resolution and low spectral resolution. On the one side these constraints limit access to specific information, like the kinetics of fast fluorescence induction or the spectrum of fluorescence emission, which would be of great help in interpreting the phenomena observed. On the other side, those limitations pushed us to have a very empirical approach, which has given however encouraging results.

In the field of fruit quality assessment, the application of chlorophyll fluorescence imaging has still been very limited (Abbott, 1996; Abbott 1999). This study represents the first extensive research on the subject. It is thus difficult to approach a discussion of the data, due to the lack of previous references.

The data presented here show that chlorophyll fluorescence imaging can be applied successfully to estimate physiological age/maturity of the fruit (section 3.3.2) and to predict development of some disorders (section 3.3.3). Effects of agricultural practice like fertilization (3.3.4) can also be detected by fluorescence imaging, though the interpretation of the results is not trivial.

A correct estimation of the maturity of an apple is very important in order to select the best harvest date and obtain a high quality product. Chlorophyll fluorescence imaging will prove to be an important tool in this field. Perhaps not at the pre-harvest level, when imaging fruits on the tree and pick them selectively would be technically challenging and most probably not effective. But at the post-harvest level fluorescence imaging would be ideally suited as an in-line application on grading lines. Such machineries, once provided with an in-line fluorescence imaging system, could grade fruits not only based on color and size, but also based on the physiological age of the fruit, which is related to its storage potential. Without the support of other physiological data it is difficult to say whether the decline in time of the fluorescence signal is due simply to breakdown of chlorophyll or to a more complex mechanism. However, such decline is evident and highly repeatable, and from an empirical
point of view it provides a practical estimation of the level of maturity of the fruit. More research is required not only to investigate the physiology behind the phenomenon, but also to verify the results of such technique with other apple cultivars and perhaps other fruits. Furthermore, a substantial amount of work will be required to analyze thousands of samples in order to establish a reference scale for the estimation of maturity based on fluorescence data.

With respect to the last point, great help will come from the use of artificial neural networks (ANNs). ANNs have been applied already in the analysis of fluorescence data (Tyystjärvi et al., 1999) and to classify agricultural products (Chen et al., 1995; Song et al., 1995). Neural network analysis of the imaging data presented in sections 3.3.4 and 3.3.5 show that it is possible to train an artificial neural network to classify apple samples based solely on fluorescence imaging data. Such a system could be integrated in embedded systems on fruit grading lines. We are aware that the recognition rates obtained in the present work are still far from values that could be acceptable from a commercial point of view. We believe that the cause of the lengthy training phase (tens of thousands of cycles) and the relatively low recognition rates resides in the small size of the sample. A desirable set of data for training the neural network would be of several thousands of apples. This is very demanding in terms of labor, but work in this direction is already in progress at the Laboratory of Botany of the Limburgs Universitair Centrum.
Chapter 4

Other applications of fluorescence imaging

The fluorescence imaging system (FIS) can be applied in a large range of research fields. Potentially, any photosynthesizing organism, ranging from unicellular algae to higher plants, could be investigated by means of this technique. During the development of the research project, although the main focus was on fruit quality assessment, a number of applications have been successfully demonstrated. These applications covered various domains, the most successful being applications in the field of plant stress research.

In this chapter I will report about some of those applications. In particular we will see how chlorophyll fluorescence imaging can be of great help in investigating the effects on plants of heavy metals, hormone unbalance and viral infections.

4.1 Heavy Metal Stress

4.1.1 Introduction

Toxic concentrations of heavy metals in the environment affect the physiology of the plant at several levels, with the photosynthetic machinery as one of the major targets (Vangronsveld and Clijsters, 1994). Early detection of an alteration of the plant health status is an important requirement for taking efficient countermeasures to prevent further damage and to recover from the stress impact.

In this work, a combination of visible-light induced chlorophyll fluorescence and UV-laser-induced fluorescence imaging was used to follow throughout the leaf, the distribution of the effect that Cd-ions exert on the fluorescence and to evaluate the physiological status of the contaminated leaf. The experimental imaging data of both techniques were evaluated and related to the response of the plants to heavy metal stress. Data reported in this section have been presented at the 13th S.P.I.E. Annual International Symposium, Orlando, FL, USA, (Valcke et al., 1999).
4.1.2 Materials and methods

4.1.2.1 Plant material

Germination of Phaseolus vulgaris L. cv. Limburgse vroege seeds was induced by chilling at 4°C during 2 days followed by incubation between humidified rock wool for 4 days. This resulted in a homogeneous germination of the bean seeds. The seedlings were then transferred to vermiculite supplemented with a half-strength Hoogland solution. After 7 days, a solution containing 50 ppm Cd was applied every two days. The first images were taken four days, the second series eight days and the last images eleven days after the start of the treatment.

4.1.2.2 Fluorescence imaging

Visible-light-induced fluorescence imaging was carried out with the imaging system described in chapter two, using the protocol “quenching continuous” (see Appendix A), in which the first image was grabbed within 1 s from the onset of constant illumination (λ < 650 nm), and then images were grabbed at time intervals of 2, 5, 10, 20, 30, 60, 90, 120, 180, 240 and 300 sec after the onset of the illumination. As an additional step in the image analysis, six regions of interest (ROI’s) of 3x3 pixels were selected for each series of corrected images to follow the time course of the fluorescence quenching. The fluorescence value of each ROI was calculated as the integral of the pixel values in the ROI. The value was then normalized dividing by the difference of the value for the same ROI at time t=1 sec and time t=300 sec in order to normalize against local differences in chlorophyll content. Best visualization of the Cd-induced alteration of the fluorescence emission was obtained from the images grabbed at 120 sec after onset of the illumination. The histogram of each of these images was clipped to eliminate background noise and stretched to improve contrast.

For the UV-induced fluorescence, the laser-based imaging system of the Groupe d’Optique Appliquée, CNRS, Strasbourg, France (see section 1.2) was used. The system is composed of three main parts: (i) excitation unit, (ii) detection module and (iii) image registration, storage and processing (fig.1b). The excitation source is a Nd:YAG laser (Spectra Physics) emitting, after third harmonic generation, UV-A (355 nm) light pulses of 10 ns width, with an energy of 15 mJ, and a variable repetition rate up to 20 kHz. The same laser after second harmonic generation emitted green light pulses at 532 nm with an energy of 45 mJ. The laser beam was directed onto the sample via a divergent lens in order to illuminate a
30 cm diameter spot at the leaf (plant) level. The detection unit (RAGM + Animater V1, ARP, Strasbourg, France) consists of a gated intensified digital CCD camera operating at 50 images/sec with a CCD (Thomson TH 7863) of 384x288 pixels array and an intensifier tube (Philips XX1414M/E) with variable gain (to 1000) and a possibility of laser synchronous gating (aperture time from 10 ns to 100 ms); entrance lenses and filter wheel with four interference filters (10 nm FWHM) centered at the plant characteristic fluorescence bands (440, 520, 690 and 740 nm); a Matrox interface card in the PC allowing real-time image visualization, image accumulation with or without ambient light subtraction, and image storage; image processing software permitting the determination of the image acquisition parameters and robust image treatment. The in vivo images of the plants were registered at a distance of 30 cm from the focusing lens of the camera and in the presence of ambient light. All the images were corrected for the non-uniformity of the excitation light and for the spectral sensitivity of the camera including the attenuation factors of the focusing lens and band-pass filters.

4.1.3 Results and discussion

As a consequence of the cadmium treatment, the only visible symptoms of toxicity were reduced growth (data not shown) and a slightly different shape of the leaf. The leaf basis in the Cd-treated plants has a kidney shape while in the control leaves the basis is more flattened (figs 4-1, 4-2). No other symptoms, like chlorosis or necrosis, were induced by the metal concentration applied in this experiment. Excitation of dark-adapted leaves with nearly saturated blue light (λ < 650 nm) resulted in a fluorescence induction kinetics curve comparable to the one already described by Ciscato and Valcke (1998). In this paper, the fast chlorophyll fluorescence induction kinetics will not be discussed. The first image taken with the fluorescence imaging technique was grabbed after 1 sec of continuous illumination. This point was recognized as representing the $F_M$ under the light conditions used. The most prominent difference in fluorescence emission is situated around 120 sec after the onset of illumination. The pictures, shown in fig. 4-1 and 4-2, represent the fluorescence emission at that time for three consecutive periods of heavy metal application. In the control series, no significant changes in the fluorescence emission can be observed. This is an indication that
during the experimental period, the influence of the natural occurring senescence is negligible. Compared to the control, a strong fluorescence at the 120 sec time period is observed in the seedlings treated with cadmium. Four days later, the 120 sec fluorescence emission signal did not change significantly. However, there are some regions exhibiting a strong fluorescence emission. This could be an indication of a local accumulation of the cadmium resulting in a firmly affected photosynthetic electron transport. Remarkably, the 120 sec fluorescence emission signal in 11-days-Cd-treated seedlings is in its global appearance not significantly different from the corresponding image of the control leaves, with the exception of some small, localized areas in which a high fluorescence emission is still present.

Figure 4-1: Control leaf. a) Color picture of the leaf taken at day 8. b) to d) Visible-light-induced chlorophyll fluorescence images: b) day 4, c) day 8 and d) day 11. Points P1 to P6 correspond to the curves shown in figure 4-3.
Figure 4-2 Cd-treated leaf: a) Black and white picture of the leaf taken at day 8. b) to d) Visible-light-induced chlorophyll fluorescence images: b) day 4, c) day 8 and d) day 11. Points P1 to P6 correspond to the curves shown in figure 4-4.

The quenching kinetics of the chlorophyll fluorescence is affected by heavy metal treatment (figs 4-3 and 4-4). The curvature is less steep so it needs more time to reach the steady state fluorescence emission. Moreover, this kinetic pattern is for Cd-treated plants, in contrast to the control leaf, different for different spots. The slowest quenching kinetic corresponds with the spot exhibiting a strong fluorescence emission. The steady state emission is for Cd-treated plants not significantly different from the control one.
Figure 4-3 Time course of the normalized fluorescence from control leaf at day 4. Curves P1 to P6 correspond to the points marked on figure 4-1.

Figure 4-4 Time course of the normalized fluorescence from Cd-treated leaf at day 4. Curves P1 to P6 correspond to the points marked on figure 4-2.

The quenching of the fluorescence emission during the fluorescence induction has been attributed to the interaction between two fundamental mechanisms, the photochemical
and the non-photochemical quenching (Havaux et al., 1991). The main component of the non-photochemical quenching is the energetic quenching reflecting the build up of a ΔpH-gradient across the thylakoid membranes (Krause and Weis, 1991). The change in kinetic behavior of the fluorescence quenching clearly indicates that due to an accumulation of cadmium, the energetic balance of the photosynthetic apparatus has been affected.

Excitation with UV-light resulted in four fluorescence-emission bands positioned in the blue (440 nm), the green (520 nm), the red (685 nm) and the far-red (730 nm) region of the visible spectrum. In contrast with the blue-light induced fluorescence, leaves were not dark-adapted and due to the low intensity of the excitation light, no induction kinetics will be induced here. The effect of a Cd-treatment is presented in fig. 4-5 in which the ratios 440/685, 440/720 and 685/720 are shown. The fluorescence emissions at 685 and 720 nm over the whole leaf area are comparable, resulting in a more or less uniform distribution of emission for the red/far-red ratio. No significant differences could be observed between the control leaf and the Cd-treated one.

Both the 440/685 and 440/720 ratios showed differences between the control and the Cd-treated plants. A higher 440/685 ratio is observed in the main vein and in some of the lateral veins located more in the middle of the leaf. Also, slight but most probably
significantly higher fluorescence ratios could be observed at the edge of the leaf in the Cd-treated plants. The higher blue/red and blue/far-red ratios in the leaves of the heavy metal treated leaves could be an indication of a higher content of secondary metabolites such as ferulic acid or coumaric acid which are involved in the lignin biosynthesis, a component of the cell wall. The increase in lignin synthesis, accompanied with a stimulation of some of the enzymes of the Shikimata-pathway has been observed when stress was applied to plants (Krause and Weis, 1991).

The data presented here clearly show the complementary of both fluorescence-imaging techniques in evaluating the effect of heavy metal induced stress. Cadmium affects seriously the photosynthetic electron transport capacity. As previously shown, cadmium has a remarkable effect on the kinetics of the fluorescence induction, especially at the level of I, the point located 2 msec after the onset of illumination (Ciscato et al., 1999). This point reflects the fraction of reduced QA and represents the capability of the re-oxidation of QA by QB. The results were interpreted in terms of plastic and elastic stress-response of the PSII-complex (Krüger et al., 1997). The visible-light induced chlorophyll fluorescence imaging adds evidence to this interpretation. The quenching of the chlorophyll fluorescence, which is a superposition of photochemical and non-photochemical events, reflects the capacity of the electron transport beyond the plastoquinone pool. The Cd-induced loss of the re-oxidation capacity of QA results in a disturbed capacity to build up an efficient pH-gradient similar to non-treated plants.

The fluorescence emission pattern induced by the UV-excitation and represented in this paper by the different ratios of the emission maxima clearly indicated that due to cadmium treatment, a response of the plant was induced in which the synthesis of secondary metabolites was involved. This induction was reflected in an increase in the fluorescence emission in the blue/green region.

The most probable accumulation of cadmium at certain spots in the leaf, characterized by a high fluorescence emission, is not clear at the moment. The results of the chlorophyll fluorescence imaging presented here are in vivo experimental evidence that the leaf is not a homogeneous structure in terms of cellular topology. This heterogeneous character of the leaf is reflected by the heterogeneous distribution of cadmium induced high chlorophyll fluorescence emission.
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The use of a non-destructive, non-invasive and real-time measurement technology like fluorescence imaging in the field of heavy metal stress is quite novel and requires further improvement. More experiments dealing with other types of heavy metal stress in combination with destructive approaches are needed in order to be able to interpret in an objective way the imaging signals. Nevertheless, the results presented in this work clearly indicate that the fluorescence imaging technology can be used as a powerful non-destructive diagnostic tool for heavy metal stress in plants.
4.2 Transgenic Pssu-ipt Tobacco

4.2.1 Introduction

Effects of endogenous cytokinin (CK) on photosynthesis are being studied at the Laboratory of Botany of the Limburgs Universitair Centrum. In the framework of this research, fluorescence emission by transgenic Pssu-ipt tobacco as compared with the wildtype has been investigated by means of the fluorescence imaging system (FIS). Ipt-transgenic tobacco is characterized by an elevated content of endogenous CK. This class of phytohormones is known to affect photosynthetic activity at various levels (Valcke and Quantem, 1999; Synková et al., 1999; Synková et al., 1997).

The FIS has been used in the study of transgenic Pssu-ipt tobacco in order to highlight possible topological and functional alteration caused by the elevated content of CK.

Preliminary data are presented here with the sole purpose of giving an example of a research topic in which the fluorescence imaging system provides new insights and represents a valuable research tool.

4.2.2 Materials and methods

4.2.2.1 Plant material

Transgenic tobacco plants (Nicotiana tabacum L. cv. Petit Havana SR1) containing a supplementary ipt-gene (Pssu-ipt) were generated by means of Agrobacterium tumefaciens as described by Beinsberger et al. (1992a).

Plants were cultivated in a greenhouse under the following conditions: temperature 18°C/25°C night/day, additional illumination 12h/day with AgroSon T (400 W) and HTQ (400 W) lamps, photon flux density (pfu) approximately 200 μE m⁻² s⁻¹. Air relative humidity (RH) was 60%.

4.2.2.2 Fluorescence imaging

Due to the large size of the plants, which made placing them into the lightproof cabinet impossible, leaves needed to be detached in order to prepare them for the measurement. The detached stem was placed into a test tube containing water to prevent dehydration of the sample. The experimental protocol “Quenching continuous + pulses” as described in appendix A was used. Only data from the first part of the experiment (“Quenching continuous”) are reported.
4.2.3 Results and discussion

The slow phase of the chlorophyll fluorescence induction kinetics was followed under continuous light ($\lambda < 650$ nm, see chapter 2) by means of the fluorescence imaging system. Images from leaves from two representative samples are reported here. One is a wildtype (WT) and the other a first generation rooted Pssu-ipt transgenic (TG) tobacco plant. Figure 4-6 shows the visual appearance of the two leaves. The TG sample presented a greener color than WT, without any particular pattern.

![Figure 4-6 Photographs of leaves of wildtype (WT, left) and Pssu-ipt transgenic (TG, right) tobacco.](image)

Figures 4-7 and 4-8 report the full sequence of fluorescence images for WT and TG respectively. In the WT leaf, fluorescence declines homogeneously over the whole leaf surface, as testified by the progressive darkening of the image with time. The behavior of the TG leaf is remarkably different. Distinct areas show different degrees of fluorescence quenching. In particular, a distinction can be made between areas surrounding the vascular system and the rest of the mesophyll. In this latter zone the decline in fluorescence is faster and more pronounced than in areas close to the veins. In figures 4-9 and 4-10, fluorescence emission vs. time as extracted from various points in the leaves is reported. The traces clearly indicate that while the areas surrounding the veins in the TG leaves have a behavior comparable to that of a WT leaf, fluorescence emitted by the dark areas in figure 4-8 shows a peculiar kinetics of fluorescence emission. In particular, there is a faster quenching and no clear secondary peaks. It is very difficult to speculate about the causes of such difference in the absence of other support data. On the other hand it is also very difficult to obtain other kinds of information (e.g. measure of rate of electron transport and the like) in such small
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areas due to the small size of the area and the implied lack of material. However, a few considerations can be given.

The elevated endogenous content of CK causes in tobacco morphological alterations and disturbances of photosynthesis (Beisenberg et al, 1992b; Synková et al., 1998). In particular, root formation is dramatically suppressed, causing an unbalance in the source/sink

Figure 4.7 Kinetics of fluorescence induction of a leaf from wildtype tobacco as measured by the fluorescence imaging system. From left to right, top to bottom, fluorescence images after 1, 2, 5, 10, 20, 30, 60, 90, 120, 180, 240, and 300 seconds under continuous excitation (see chapter 2 for further experimental details).
relationship and alteration of the water status. Stomatal conductance has been reported to be largely reduced in Pssu-ipt plants (Synková et al., 1999). The altered kinetics of fluorescence emission could then be ascribed to a combination of various factors. It seems unlikely that the changes in stomatal conductance alone could justify the pattern observed, as the areas with lower fluorescence are well defined in specific zones (vein surroundings) and not patchily distributed.

Figure 4-8 Kinetics of fluorescence induction from a leaf of Pssu-ipt transgenic tobacco as measured by the fluorescence imaging system. From left to right, top to bottom, fluorescence images after 1, 2, 5, 10, 20, 30, 60, 90, 120, 180, 240 and 300 seconds under continuous excitation (see chapter 2 for further experimental details).
Figure 4-9 Kinetics of fluorescence induction extracted from the point P1-P4 marked in figures 4-7 and 4-8. WT = wildtype. TG = Pssu-ipt transgenic tobacco.

Figure 4-10 Kinetics of fluorescence induction extracted from the point P1-P4 marked in figures 4-7 and 4-8. WT = wildtype. TG = Pssu-ipt transgenic tobacco. Fluorescence values normalized as \((F_t - F_{300})/(F_0 - F_{300})\)

The association of the fluorescence emission pattern with the vascular system could imply an effect of a localized situation of water stress as perhaps due to a shortage of water supply as a consequence of the morphological changes in the root system. Areas further away from the main veins could be in a condition of experiencing water shortage. In order to confirm or reject such hypothesis, it would be interesting, though technically not trivial, to associate fluorescence imaging with localized measurement of the water status of the

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mesophyll.

A direct effect of CKs on the photosynthetic apparatus also could not be excluded. A reduced electron transport capacity has been observed in transgenic Pssu-ipt tobacco as compared to control plants (Synková et al., 1998). However, such effect could not explain the peculiar localization of the altered fluorescence emission, unless differential levels of expression of the ipt-gene are assumed, but there is currently no evidence to support such a view.
4.3 Fluorescence Imaging in the study of plant-virus interaction

4.3.1 Introduction

Chlorophyll fluorescence has been used before to study plant interactions with several biotic pathogens at the level of the photosynthetic apparatus. Lesions in photobioenergetic processes may cause a reaction in the plant, which activates photoprotective mechanisms to cope with excess excitation. Eventually, the reaction may evolve into photo-inhibitory processes. In both cases the balance between photochemical and non-photochemical processes is altered, which in fact affects the emission of chlorophyll fluorescence (Balachandran et al., 1997). Thus, chlorophyll fluorescence imaging provides a very convenient tool to investigate early plant-virus interaction.

This section presents preliminary data from experiments conducted in cooperation with the group of Dr. Matilde Barón Ayala (Estación Experimental del Zaidín, CSIC, Granada, Spain) on *Nicotiana benthamiana* plants inoculated with a mild suspension of an Italian strain of pepper mild mottle virus (PMMoV-I).

4.3.2 Materials and methods

4.3.2.1 Plant material and inoculation

*Nicotiana benthamiana* plants were grown under controlled environmental conditions in a greenhouse: temperature 18°C/25°C night/day, additional illumination 12h/day with AgroSon T (400 W) and HTQ (400 W) lamps, photon flux density (pfd) was approximately 200 μE m⁻² s⁻¹. Air relative humidity (RH) was 60%. After three weeks the young plants were transferred to a growth chamber with the following conditions: temperature 17°C/22°C night/day, 16 hours photoperiod with pfd of 200 μ E m⁻² s⁻¹ provided by fluorescent tubes and incandescent lamps, RH=80%. After two more weeks the plants were inoculated with a mild suspension of an Italian strain of pepper mild mottle virus (PMMoV-I) in Na-phosphate buffer pH 7. Viral suspension was provided by Dr. Luque of the Centro de Investigaciones Biológicas (CSIC, Madrid, Spain).

Inoculation was performed on the three older leaves of each plant. 50 μl of a solution of 50 μg of virus/ml were dropped on the leaves and the leaves were gently rubbed after
dusting them with abrasive Carborundum powder. This procedure had the aim of scratching the epidermis so as to let the virus penetrate the leaf tissue.

Observations were done at 7, 14 and 21 days post-inoculation (d.p.i.).

4.3.2.2 Fluorescence imaging

For chlorophyll fluorescence imaging, control and inoculated *Nicotiana benthamiana* plants were dark adapted for at least 15 min in a closed cabinet. The minimal dark adaptation time was determined in advance by measuring the quantum yield of photochemistry ($F_v/F_M$) after increasing dark-adaptation periods, and choosing the minimal time necessary for $F_v/F_M$ to reach its maximum value; the Plant Efficiency Analyser (PEA, Hansatech, Norfolk, UK) was used for this purpose. After dark adaptation, the whole plant was placed inside the instrument’s cabinet and a leaf was laid onto the ring-shaped holder equipped with a plastic grid to support the sample. These two operations were carried out in darkness, in order to preserve the status of the sample. Fluorescence imaging was carried out following the “Quenching continuous + pulses” as described in appendix A. Measurements were performed on both young and mature leaves, but never on the leaves used for the inoculation, as they had been damaged by the process. After measuring inoculated plants, parts of the instrument and other tools that had been in contact with the plant were disinfected with sodium hypochlorite in order to avoid spreading the infection to other plants.

4.3.3 Results and discussion

The series of experiments, from which only a little part is reported here, was planned in order to follow by means of chlorophyll fluorescence imaging the development of the viral infection and related symptoms. Experimental work in this field is still in progress and only one striking example will be presented here, in which the fluorescence imaging could highlight a peculiar 2-D pattern of altered kinetics of the fluorescence emission. Imaging was performed on both asymptomatic and symptomatic leaves. The samples shown here are the youngest fully developed asymptomatic leaf of an inoculated plant, and a comparable leaf of a control plant. The pattern found in the inoculated plant reported here was observed in several infected plants but in none of the control ones.
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Figure 4-11 NPQ30 (left) and NPQ (right) images of a *Nicotiana benthamiana* leaf from a control plant.

Figures 4-11 and 4-12 show in false colors the NPQ and NPQ$_{30}$ images of the control and the infected leaf respectively. NPQ stands for non-photochemical quenching. It is a measure of non-photochemical energy dissipation and is calculated as $(F_M - F'_M)/F_M$ (Scholes and Rolfe, 1996) where $F_M$ is the maximal fluorescence (at $t = 1$ sec in our case) and $F'_M$ is the fluorescence measured during a saturating pulse at the steady state (300 sec). The 30 in the subscript indicates that the $F'_M$ used is the one measured after 30 sec from the onset of the illumination. The reason why we used also the NPQ$_{30}$ is that the effect is most evident at this moment.

Figure 4-12 NPQ30 (left) and NPQ (right) images of an asymptomatic *Nicotiana benthamiana* leaf from an infected plant.

The effect we are talking about is the appearance of a sharp dark edge marking the border between the surroundings of the main veins and areas of the mesophyll with higher NPQ. It could be speculated that such an effect is linked to the spreading of the virus through
the vascular system. Brighter areas represent zones of higher energy dissipation, while the dark line could indicate a bordering zone where the tissue has a first contact with the virus. In this bordering zone we could expect an increased photosynthetic activity, correlated to the decreased non-photochemical energy dissipation. Images of the photosynthetic activity (figure 4-13) calculated according to Genty and Meyer (1994) as $F_M' - F_S/F_S$ (where $F_S$ = fluorescence at the steady state), unfortunately, do not support this hypothesis. No particular pattern was observed in either the control or the infected plant, though the latter seems to show reduced activity. However the use of this parameter in our experimental condition, i.e. with light pulses of not extremely high intensity, is questionable.

The reason why the effect on the NPQ is more evident after 30 sec than at the steady state can probably be found in a different kinetics of fluorescence emission in different areas of the leaf.

![Figure 4-13](image)

*Figure 4-13* Images of the photosynthetic activity of leaves from control (left) and inoculated (right) *Nicotiana benthamiana* plants. Photosynthetic activity is calculated according to Genty and Meyer (1994) as $F_M' - F_S/F_S$.

As pointed out in the introduction of this section, the data presented here are only preliminary, so no further discussion is in order at the moment. Once again, fluorescence imaging represents an excellent tool for the investigation of stress effects on the photosynthetic apparatus also in the field of biotic stress. However, before we can rely on the results given by the technique, more experimental work needs to be done in order to validate the results obtained.
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Appendix A – The Grabix software

Grabix is the software application developed by the author to automate capturing of images and synchronization with operation of the illumination system.

In its early version, the instrument was controlled by a Sun SparcClassic workstation running the Solaris 2.4 operating system. The software was simply called “Grab” and consisted of a script written in C-shell language that integrated a program written by the author to drive the lamps via a custom-made interface connected to the serial port of the computer, and a program to capture images and save them in Sun raster format. The latter image-capturing program was supplied by the manufacturer of the frame grabber card (EDT S1V, Engineering Design Team Inc., Beaverton, OR, USA).

![Grabix Software Interface](image)

**Figure A-1** Screenshot of the main window of the software for fluorescence image capture Grabix 2000.

At a later stage, the system was ported to an Intel platform running the Linux operating system (distribution Slackware 7.0, kernel 2.2.13) and the software rewritten. Its new version – called “Grabix” – was written in Tcl/Tk and supplied with a graphical user interface (fig. A-1) running under the X-Windows environment. The application is composed of two modules written in C language. The first one – “digout” – drives the lamps via an AD/DA converter; it operates the converter board by direct hardware addressing. The second one – “grab_raw” – grabs images and stores them in raw integer format; it is based on the Video4Linux API. A version of the capture software that grabs gray scale images and saves them in 8-bit PGM format is also available. The Tcl/Tk scripting language has been chosen because its easy to use, as compared to compiled languages like C, yet allowing the development of fully featured user-friendly graphical user interfaces. More details about Tcl/Tk can be found at [http://dev.scriptics.com/software/tcltk/](http://dev.scriptics.com/software/tcltk/)
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Several versions of Grabix have been released, differing in the number of customizable parameters. A view of Grabix's latest version – named "Grabix 2000" – is presented in figure A-1. On the left panel the user can select the desired imaging protocol: "Quenching continuous" for quenching analysis in continuous high light, "Quenching pulses" for quenching analysis in continuous actinic light and high intensity pulses, and "Rfd" for the Rfd analysis in continuous high light (Lichtenthaler and Rinderle, 1988). In the central panel the user can insert the parameters required by the various protocols of analysis. More on the right there are switches to manually operate the lamps. The right panel is where the user must insert the directory name and the file name for the sample to be analyzed. Also on the right panel there are buttons to start the procedure ("Start"), grab single images ("Single hit") or double images ("Double hit"), i.e. two images in sequence, one in darkness or actinic light and one after 1 second in full light. This latter procedure was designed to capture either simply F_M images, or images of F_S and F'_M with which one can calculate the quantum yield of photochemistry according to Genty et al. (1995).

The source code and further details about Grabix and its development are available upon request from the author (mciscato@luc.ac.be).
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Appendix B – Other Software Tools

B1 – Khoros Pro 2001

![Figure B-1 Screenshot of the Cantata visual programming environment of the Khoros system. Each icon (glyph) represents an image processing operation. Arcs connecting glyphs visualize the data flow.](image)

Khoros Pro 2001 is a product of Khoral Research Inc. It is a very complete and flexible suite of programs for image processing and analysis. It runs under a number of platforms and operating systems. A free version running under the UNIX operating system (including Linux) is available free of charge for students at [http://www.khoral.com](http://www.khoral.com)

For the work presented here, Khoros has been used mainly through its visual programming environment Cantata (fig. B-1). Cantata provides a graphical user interface in which icons (called “glyphs”) represent image processing operations and lines connecting the
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glyphs represent the data flow. Figure B-1 shows the procedure ("workspace") used to process apple images according to the protocol described in section 3.2.2. Data flow is from left to right. When necessary count loops can be created, for example to process automatically large sets of images. Khoros can import and export images from and to a wide variety of file formats. In our case unprocessed files are imported as raw unsigned integer data and exported in text (ASCII) form.

Advanced features of the Khoros system include coding of custom image processing programs and compilation of procedures into stand-alone programs. Such features have not been used in the present work and will not be covered here.
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B2 – The R statistical analysis package

R is a language and environment for statistical computing and graphics. It is available as Free Software under the terms of the Free Software Foundation's GNU General Public License in source code form. With R it is possible to import data from various formats, and process them by means of the numerous functions available with the package or as contributed packages. Being a full featured programming language, it allows also developing custom functions, though this feature has not been exploited in the present project. More information and the software itself can be found at http://cran.r-project.org/

In the present work R has been used to perform statistical analysis of the fluorescence images. In a typical experiment, the final product of the image processing is an

```r
# Create the empty result matrix with titles
resultT1 <- matrix(data = NA, nrow = 0, ncol = 5, dimnames = list(NULL, c("R-nr.", "1%", "5%", "25%", "Median", "75%", "95%", "99%", "Mean", "sd", "interc.", "slope", "R^2", "G-nr.", "1%", "5%", "25%", "Median", "75%", "95%", "99%", "Mean", "sd", "interc.", "slope", "R^2")))

# Process the first 9 images
for(n in 1:9){
  print(n)

  # Create vector for red side image
  tR <- sort(scan(paste("e:/PhD-data/apple-oct-99/JN/J111/J111-0",n,".R.asc","sep="")))[14106:65536]
  normtR <- qnorm(1:51431/51432,mean=mean(tR),sd=sd(tR))
  lmR <- lm(normtR)
  sR <- summary(lmR)
  Reside <- c(n.quantile(sR,c(.01,.05,.25,.50,.75,.95,.99)),
             mean(sR$sr),sd(sR$sr),sR$sr$squared)

  # Create vector for green side image
  tG <- sort(scan(paste("e:/PhD-data/apple-oct-99/JN/J111/J111-0",n,".G.asc","sep="")))[14106:65536]
  normtG <- qnorm(1:51431/51432,mean=mean(tG),sd=sd(tG))
  lmtG <- lm(normtG)
  sG <- summary(lmtG)
  Gside <- c(n.quantile(sG,c(.01,.05,.25,.50,.75,.95,.99)),
             mean(sG$sr),sd(sG$sr),sG$sr$squared)

  # Merge the vectors for the two sides and store the resulting vector in
  # the result matrix
  resultT1 <- rbind(resultT1,c(Reside,Gside))
}

# Write out the resulting matrix to a text file
```

Figure B-2 Example of R code used to create vectors to be used with the neural network analysis. See text for explanation.
image saved in the ASCII file format (see chapter 2 for details). This means that the image is saved on disk in form of a text table containing the pixel values of the image. The data in such a format can be imported into R either as a table, hence conserving the spatial information, or in the form of a vector composed of all pixel values. In the latter procedure normally the spatial information is lost (though it is not necessarily so), and the set of fluorescence data (i.e. the pixel values) can be used for further statistical analysis. Figure B-2 shows a piece of code used to create the vectors that have been used for the neural network analysis of apple fluorescence images, as described in section 3.2.3. Highlighted in bold red are the key functions used in the program.

- `scan()` Used to import the image as vector.
- `qnorm()` Creates a normal distribution with same mean and standard deviation as the real distribution.
- `lm()` Linear model. Performs a linear regression between the real and the simulated distributions.
- `quantile()` Calculates the quantiles specified as parameters to the function. In this case: 1% 5% 25% 50% 75% 95% and 99%
- `mean()` Returns the mean of the distribution.
- `sd()` Returns the standard deviation of the distribution.
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B3 – Stuttgart Neural Network Simulator (SNNS)

Figure B-3 A screenshot of the Stuttgart Neural Network Simulator. The window on the right shows a backpropagation network like the one used in the experiments described in chapter 3. The graph on the left shows the error function after training of the network. The other windows show various controls.

The Stuttgart Neural Network Simulator (SNNS) is a simulator for neural networks developed at the Institute for Parallel and Distributed High Performance Systems (IPVR) of the University of Stuttgart. It provides an efficient and flexible simulation environment for artificial neural networks. SNNS works under various versions of the UNIX operating system, including Linux. It is free software (though there are some restrictions to its distribution) and can be downloaded via anonymous ftp from:

ftp.informatik.uni-stuttgart.de

SNNS can simulate a wide variety of artificial neural networks. All operations and configurations are controlled via a graphical user interface (fig. B-3). Data are given to the simulator in the form of text (ASCII) files. The file for a training data set is typically composed of a standard header, followed by an alternate sequence of input and corresponding
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output vectors. The number of input and output units is specified in the header of the file. The file for a test data set is similar to the one for a training data set, with the only difference that there are no output vectors defined.

In a typical experiment a network is created with a specific tool or it is loaded from the disk. Previously created training and test data files are also loaded from the disk. After initialization of a number of parameters (activation function, number of cycles, etc.) the training procedure is started. During this phase, the training data set is used. The training phase lasts for a number of cycles selected by the user. The evolution of the error function during learning can be followed on a specific graphical window. After a number of training cycles, when the error function has reached a satisfactorily low value, the network is trained. At this point, a set of unknown data is used to test the success of learning. The results are exported to a text file, which can be used for further analysis.
References


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