Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Colture arboree ed agrosistemi forestali, ornamentali e paesaggistici

_____

Ciclo XXII

Settore scientifico-disciplinare di afferenza: AGR/05

TITOLO TESI

NOVEL TECHNIQUES FOR THE REMOTE SENSING OF PHOTOSYNTHETIC PROCESSES

Presentata da: Ammar Dayyoub

Coordinatore Dottorato **Relatore**

Prof. Luca Corelli Grappadelli **Prof. Federico Magnani**

Esame finale anno 2011

Table of Contents

Acknowledgements

I want to express my appreciation and sincere gratitude to my advisor ***Professor Fderico Magnani*** *(Professor in the faculty of agriculture-University of Bologna-ITAY), for guidance, supervision, encouragement and his great help in reviewing the manuscript.*

My deep appreciation is expressed also to ***Professor Luca Corelli Grappadelli****, the PhD coordinator in the department of Colture Arboree.*

I am also grateful to the staff of the department of Colture Arboree, with special mention to the director ***Professor Guglielmo Costa****, for giving me the possibility to complete this study.*

Sincere thanks are extended to all those who helped me and contributed to complete this work, especially ***Dr. Lucia Cantoni, Dr. Enrico Muzzi, Dr. Maurizio Ventura*** *for their cooperation, help and courtesy.*

I thank also the ***Erasmus Mundos External Cooperation*** *for the financial support of my PhD period.*

Finally sincere thanks to my **wife, family, friends** and **colleagues** for their continuous encouragements.

Ammar Khaled DAYYOUB

March 2011

NOVEL TECHNIQUES FOR THE REMOTE SENSING OF PHOTOSYNTHETIC PROCESSES

Abstract

Remote sensing (RS) techniques have evolved into an important instrument to investigate forest function. New methods based on the remote detection of leaf biochemistry and photosynthesis are being developed and applied in pilot studies from airborne and satellite platforms (PRI, solar-induced fluorescence; N and chlorophyll content), but much remains to be done for the functional interpretation of the resulting information..

Non-destructive monitoring methods, a direct application of RS studies, are also proving increasingly attractive for the determination of stress conditions or nutrient deficiencies not only in research but also in agronomy, horticulture and urban forestry (proximal remote sensing).

In this work I will focus on some novel techniques recently developed for the estimation of photochemistry and photosynthetic rates (and gross primary productivity at canopy scale), based (i) on the proximal measurement of steady-state chlorophyll fluorescence yield (as a preliminary step for the remote detection of fluorescence radiance) or (ii) the remote sensing of changes in hyperspectral leaf reflectance, associated to xanthophyll de-epoxydation and energy partitioning, which is closely coupled to leaf photochemistry and photosynthesis. I will also present and describe the thorough test of a mathematical model of leaf steady-state fluorescence and photosynthesis recently developed in our group. Two different species were used in the experiments: Arbutus unedo, a schlerophyllous Mediterranean species, and Populus euroamericana, a broad leaf deciduous tree widely used in plantation forestry throughout Europe.

Results show that ambient fluorescence could provide a useful tool for testing photosynthetic processes from a distance. These results confirm also the photosynthetic reflectance index (PRI) as an efficient remote sensing reflectance index estimating short-term changes in photochemical efficiency as well as long-term changes in leaf biochemistry.

The study also demonstrated that remote sensing techniques, whilst conveniently calibrated, could provide a fast and reliable method to estimate photosynthetic pigment content and total nitrogen, beside assessing the state of photochemical process in our plants' leaves in the field. This could have important practical applications for the management of plant cultivation systems, for the estimation of the nutrient requirements of our plants for optimal growth.

Chapter 1

General introduction

1.1. Remote Sensing: definition, components and possible applications

Remote sensing (RS) can be defined as the detection of the quantitative properties of a distant object, without any direct contact with it. It is generally based on the analysis of the reflection (or emission) by the object itself of an electromagnetic radiation, either natural (passive remote sensing) or artificially shed on the object for this particular purpose (active remote sensing).

Remote sensing is comprised of two distinct activities: on the one hand, the term refers to the activity of collecting data by sensors designed to detect electromagnetic energy from positions on ground-based, aerial, and satellite platforms; on the other hand, it also identifies the methods of interpreting those data.

Starting around 1960 by remote sensing reference is generally made to the numerical analysis of images, in contrast with photogrammetry, which is imagery (or visually)-based.

The first known forestry remote sensing application was recorded in the *Berliner Tageblatt* of September 10, 1887 (Spurr, 1960). The notice concerned the experiments of an unnamed German forester who constructed a forest map from photos acquired from a hot-air balloon. Nowadays the remote sensing of forests, vegetation and other natural resources is attracting increasing attention, in what is generally referred to as Earth Observation (EO).

Possible EO applications include (Wynne and Carter 1997): forest cover / type characterization, determination of forest stand conditions and forest health, species identification, site characterization, fire monitoring, detection of deforestation, estimation of biomass and productivity, biodiversity monitoring.

Other possible uses include the collection of data on dangerous or inaccessible areas, natural resources management, agricultural applications such as land usage and conservation and precision farming, national security and overhead, ground-based and stand-off collection on border areas, military collection of data about dangerous border areas, and medical imaging.

Any system for the acquisition of RS data includes the following components (see Fig. 1 below):

A. A source of light illuminates the object of study.
B. The incident radiation (and the reflected one) interacts with the atmosphere.
C. The incident radiation interact (is reflected) with the object, as a function of its characteristics (structural and biochemical)
D. The radiation reflected from the object is detected by a sensor on a satellite (or airplane)
E. The signal is transmitted to a ground station and registered.
F. The signal is corrected, interpreted and analyzed
G. Final practical application of the sensed data.

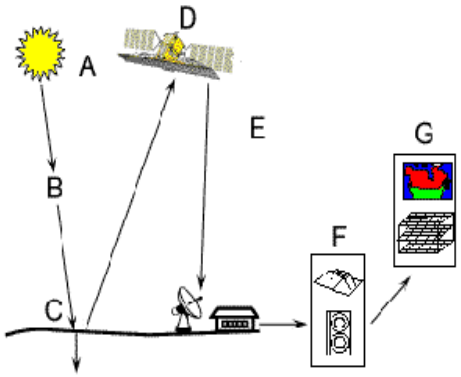

Fig. 1 Main steps of the remote sensing approach. See text for details.

1.2. Physical and biophysical background of Remote Sensing

The electromagnetic radiation (from ultraviolet to infrared) can be described alternatively as a flux of photons (a unity of energy) or as an electromagnetic

wave of frequency ν. The electromagnetic wave can also be described in terms of its wavelength λ, since it holds:

$$C = \lambda\nu \text{ (transposable to } \nu = C/\lambda \text{)}$$

where C is the speed of light.

The electromagnetic radiation extends beyond the interval of the visible light (VIS, 380-750 nm) perceived by the human eye. At lower wavelengths we distinguish the ultraviolet radiation (UV) and then X rays and Gamma rays. At longer wavelengths we have the near infrared radiation (NIR), short-wave infrared (SWIR, 750-3000 nm), medium- and far infrared (3-20 μm). At even longer wavelengths we have the microwaves (MW) and the radiowaves.

According to Planck's Law, the energy of the photon is proportional to its frequency ($E = h\nu$), *so that* ultraviolet and blue photons will be more energetic than red or infrared ones.

The radiation interacts with bodies (solid, liquid and gas) by different mechanisms:

- **transmission** (τ) some of the light that is incident on the surface of the object passes through the object.
- **refraction**, by which the photon is deflected in the passage through bodies of different density;
- **reflection** (ρ). The reflection of the light in the visible determines what we generally define as "color" (although the color perceived by the eye is also a function of the wavelength spectrum of incoming radiation);
- **scattering**, it is the case of the reflection by small particles (typically in the atmosphere);
- **absorption** (α) by molecules in the body (e.g. pigments in the leaf, water);
- **emission** in the thermal infra-red, function of the temperature of the body;
- **fluorescence** (*f*) is a (small) re-emission at longer wavelengths (lower energy) by excited molecules (e.g. chlorophyll; see below).

1.3. Leaf and vegetation reflectance

The reflectance of leaf tissues presents typically two main characteristics (Woolley 1971): a relatively high reflectance in the green spectral region, and a sharp increase of leaf reflectance between the region of the red and the near-infrared (*red-edge;* Horler et al 1983). Although reflectance in the near-infrared is typically much higher than in the green region, this is not perceived by the human eye as it occurs at wavelengths beyond its range of sensitivity. The reflectance of the leaf in the region of visible light is linked to the absorption by photosynthetic pigments (mainly chlorophyll a and b and carotenoids) of red and blue light (Taiz & Zeiger 1998), but not of green light which is therefore largely reflected or transmitted.

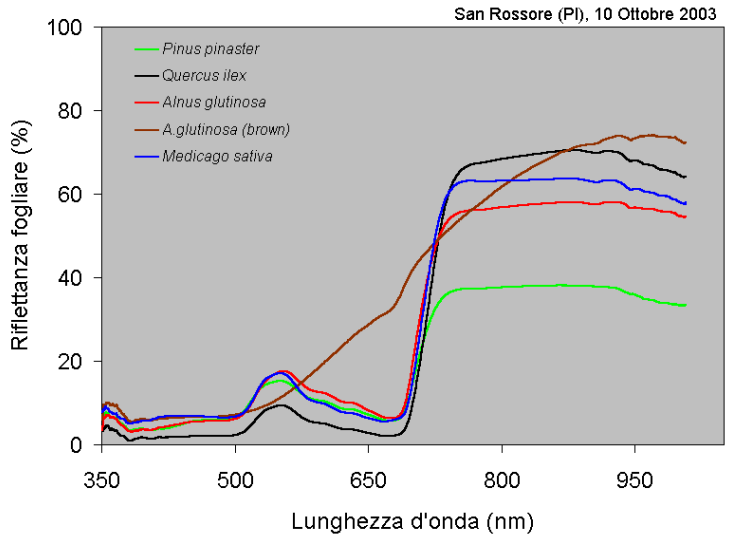

Fig. 2 Typical reflectance spectra in the VIS-NIR region for leaves of Mediterranean species acquired with a portable Ocean Optics USB-2000 spectrometer under controlled light conditions; note the marked difference between green and senescing leaves.

As a result, the changes in leaf chlorophyll content during the autumn involve an increase in leaf reflectance in the red and the orange, determining the brown color of the senescent leaf. In other cases the autumn coloring is linked also to the existence and to the increase of other compounds (oxidized phenols, anthocyanin, hydroxykynurenic acid…). In general, the reflectance of the leaf in

the visible (the “color” of the leaf) is a precise indicator of the biochemical content of the leaf.

The high reflectance observed in the near-infrared region, on the contrary, is generally assumed to be the result of light reflection at the liquid-gas interface in leaf parenchyma (Lacaze and Joffre 1994), although recent studies suggest a direct or indirect effect of leaf nitrogen content (Ollinger et al. 2008). Whatever the reason, near-infrared reflectance is known to be higher in broadleaf than in coniferous species.

1.4. The carbon balance of vegetation

Vegetation gross primary productivity (*GPP*) can be defined as the sum of photosynthesis over all the leaves in the canopy. It is the primary determinant of plant growth and C sequestration by vegetation, although it is just the first step towards the quantification of the carbon cycle. The overall flux of C at the ecosystem scale (*NEP*, net ecosystem production) is also a function of the respiration by autotrophic green plants (R_a) and by heterotrophs (R_h):

$$NEP = GPP - R_a - R_h$$

Gross primary production is primarily a function of the total amount of radiation absorbed by the canopy (*APAR*, absorbed photosynthetically active radiation), which will depend upon *PAR* (photosynthetically active radiation) and the fraction that is absorbed by the canopy (*fAPAR*, itself a function of canopy closure and leaf area index, *LAI*):

$$GPP = \varepsilon \cdot APAR = \varepsilon \cdot PAR \cdot fAPAR$$

In contrast with the curvilinear photosynthetic response to light at the leaf level, a linear relationship is generally observed at the canopy level, as a result of internal leaf shading and optimal biochemical acclimation (Dewar 1996). The conversion factor known as radiation-use efficiency (*RUE*, or ε) provides a means to estimate the rate of photosynthesis (or biomass accumulation) per land area (Monteith 1972). Both ε and *fAPAR* can be estimated by remote sensing techniques, as

discussed below; although remote sensing has traditionally focused on the estimation of *fAPAR* and light interception, photosynthetic light-use efficiency cannot be assumed to be constant in space and time, but changes with species, site fertility and environmental conditions. Recent advances in remote sensing have therefore addressed its estimation from satellite images.

At larger spatial and temporal scales (10-several 10^6 km^2 and decades or centuries), net biome productivity (*NBP*) is a more appropriate variable, which also includes the disturbance flux (*D*) which relates to the rate of change in disturbance and land-use change and can be estimated from broad band remote sensing.

$$NBP = NEP - D$$

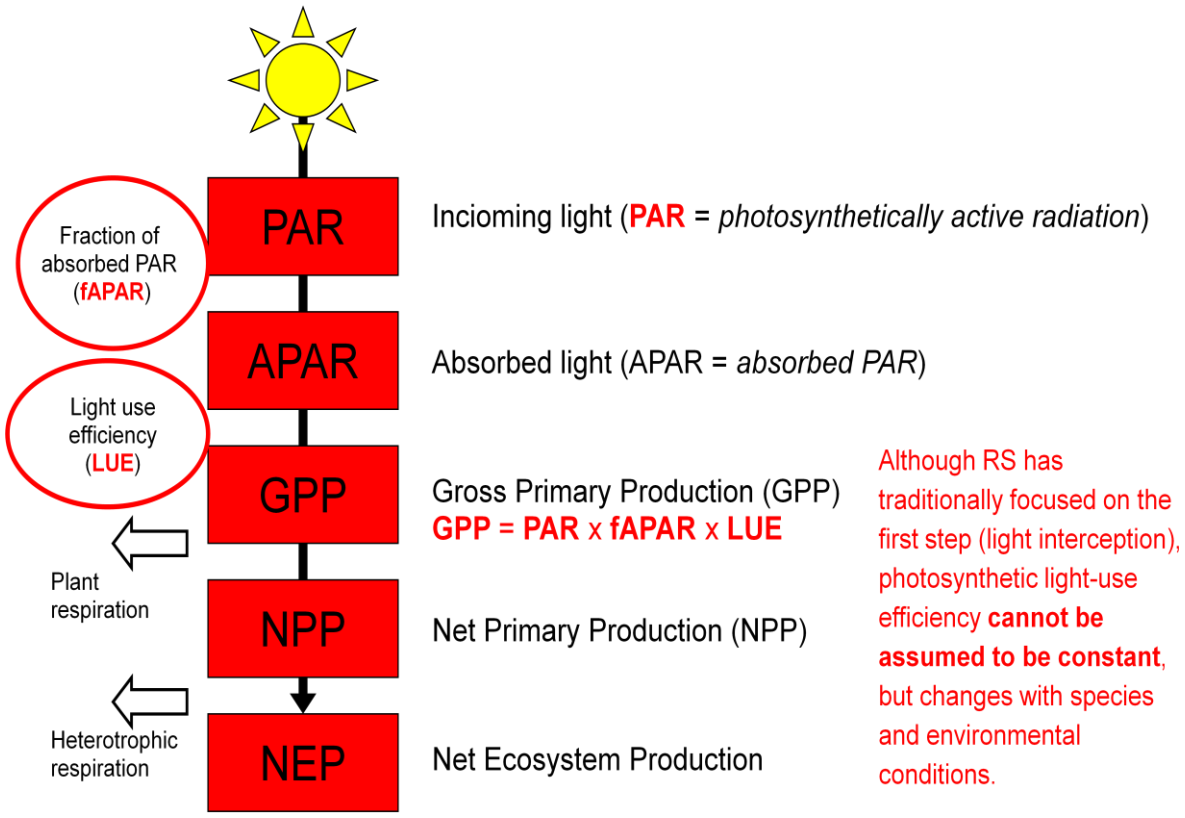

Fig. 3 A schematic diagram of the C balance of vegetation, in terms of energy capture and transformation.

1.5. Estimation of fAPAR by remote sensing techniques

The fraction of light absorbed by a canopy can be estimated from space through the Normalized Difference Vegetation Index (NDVI), based on canopy reflectance at two wavelengths in the red (R_{red}) and near infra-red (R_{nir}) parts of the solar spectrum:

$$NDVI = \frac{R_{nir} - R_{red}}{R_{nir} + R_{red}}$$

This index is essentially a measure of 'greenness': it has been used to estimate the leaf area per unit of land area (LAI) but it bears a near-linear relationship with the fraction of absorbed photosynthetically active radiation (*fAPAR*) (Myneni and Williams 1994; *Gamon et al. 1995)*.

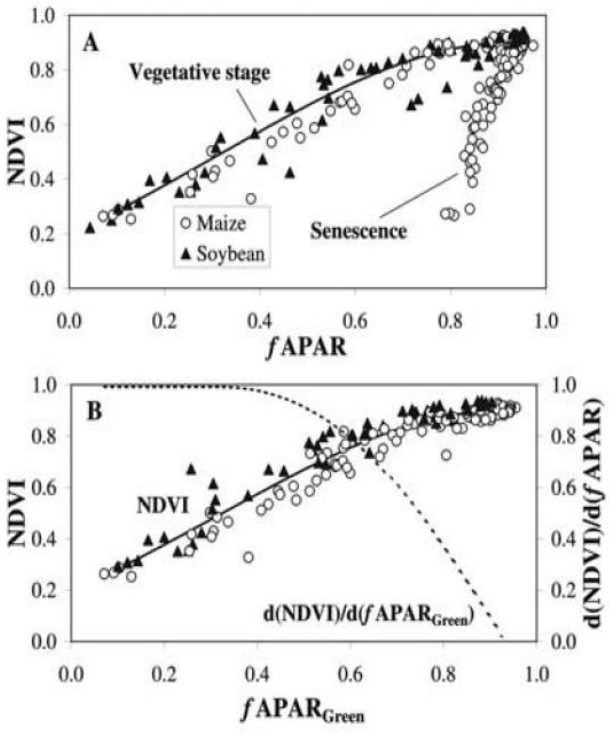

Fig. 4 Relationship NDVI vs. *f*APAR (A) and NDVI vs. *f*APARgreen (B) for irrigated and rainfed maize and soybean. Dottet line in (Fig. 4B) is the first derivative of the best fit polynomial function of NDVI vs. *f*APARGreen with respect to *f*APARgreen (Vina and Gitelson 2005).

1.6. The functional basis of LUE estimation by remote sensing techniques

A photon intercepted by a chlorophyll molecule in the leaf chloroplast can undergo several distinct fates (see Fig. 5), as the excited singlet chlorophyll molecule can dissipate its energy by four different mechanisms: an electron can be stripped from a donor molecule, initiating electron transport and photochemistry, the primary step of photosynthesis; alternatively, energy can be dissipated as heat, a process up-regulated by any limitations of photosynthetic dark reactions; finally, a small fraction of absorbed energy is re-emitted by chlorophyll at longer wavelengths, a process known as chlorophyll fluorescence (Demmig-Adams and Adams 2000). The formation of chlorophyll triplets, leading to the formation of singlet oxygen and photo-oxidative damage, is a rarer event under normal conditions.

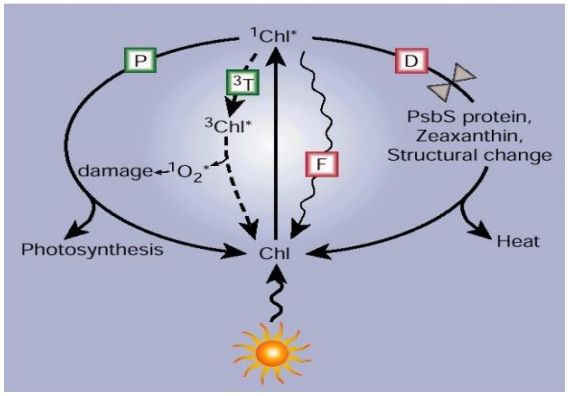

Fig. 5 Possible fates of intercepted photons. Chl chlorophyll, ^{1}Chl* excited singlet chlorophyll, ^{3}Chl* excited triplet chlorophyll, P photochemistry (green), D safe dissipation of excess excitation energy as heat, F fluorescence, ^{3}T triplet pathway, leading to the formation of singlet oxygen ($^1O_2$*) and photo-oxidative damage (Demmig-Adams and Adams 2000).

Being the main processes involved, photochemistry and thermal energy dissipation are inversely related, and can be (in first principle) estimated the one from the other.

The dissipation of excess energy as heat is known to be the result of the interactions between chlorophyll and leaf xanthophylls in photosystem II (PSII). Under high light conditions, electron transport results in a proton build-up in the thylakoid lumen and lumen acidification. Apart from driving ATP synthesis, low lumen pH results in the protonation of the carboxyl residues on the minor LHCII (light-harvesting complexes) and in the parallel de-epoxidation of the xanthophyll pigment violaxanthin to zeaxanthin. Zeaxanthin can then bind to the protonated carboxyl residues on LHCII, resulting in a conformational change (increased proximity of either Chls or xanthophyll and Chl) and eventually in an increase in energy dissipation as heat (so-called non-photochemical quenching), which is associated with a change in organization of LHCII (Horton et al. 1994).

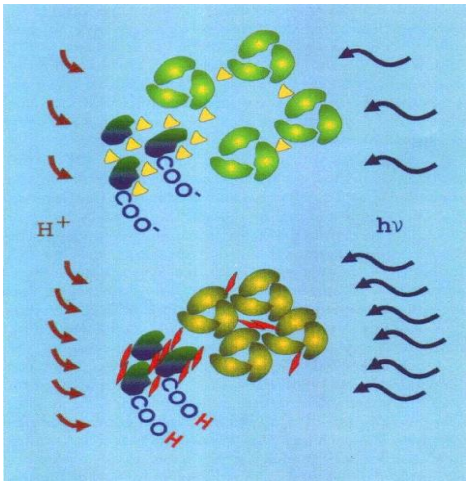

Fig. 6 Changes in PSII LHC associated with energy dissipation as heat change from low light (top) to high light (bottom) results in increased proton concentration (red arrows) in the thylakoid lumen (lumen acidification). Protonation of the carboxyl residues on the minor LHCII (light-harvesting complexes; blue/green) and de-epoxidation of the xanthophyll violaxanthin (yellow) to zeaxanthin (red) results. From (Horton et al. 1994).

Xanthophylls are auxiliary pigments, which can be found in three easily inter-convertible forms (zeaxanthin, violaxanthin and the intermediate form antheraxanthin); the de-epoxidation of violaxanthin to zeaxanthin (via

antheraxanthin) is a reversible process driven by changes in lumen pH. This typically reflects in diurnal cycles of zeaxanthin content, in response to changes in light and lumen pH, which result in a corresponding pattern in energy dissipation as heat (non-photochemical quenching).

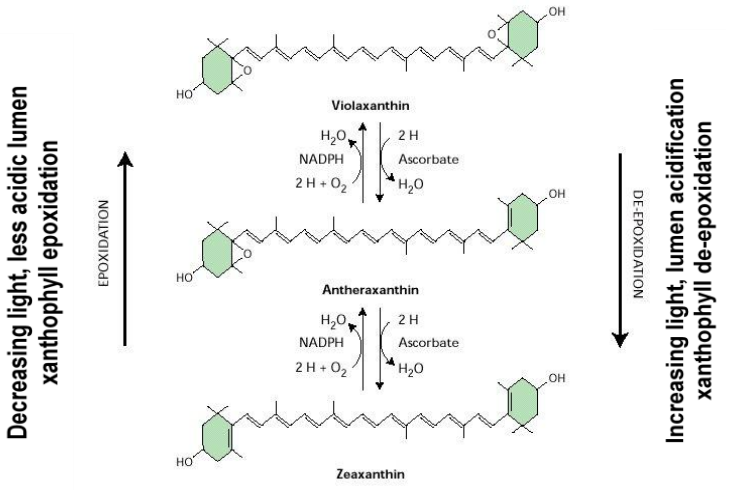

Fig. 7 Molecular structure of xanthophylls and schematic representation of xanthophylls epoxidation. From Taiz & Zeiger (1998).

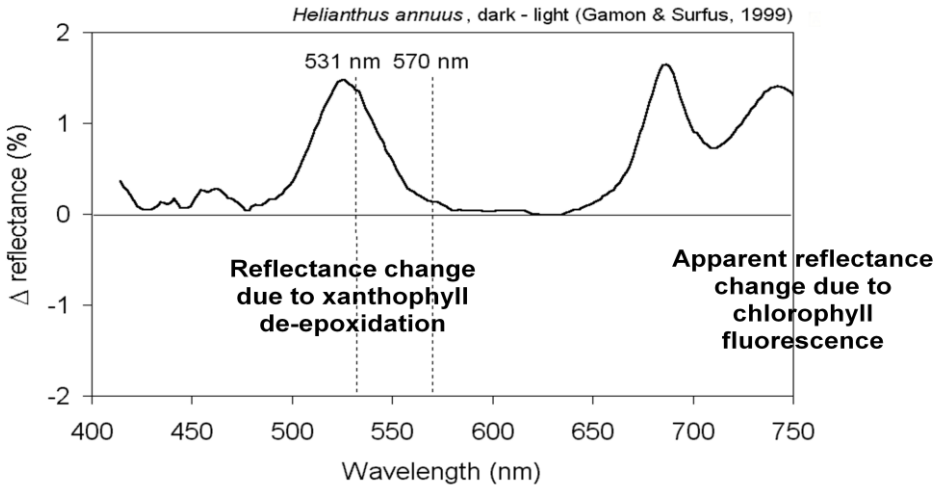

Fig. 8 Changes in the apparent reflectance spectrum of a sunflower (*Helianthus annuus* L.) dark-acclimated leaf in response to a transition to full light. The two evident features can be attributed to xanthophylls de-epoxidation and conformational changes (at 531 nm) and to chlorophyll fluorescence (at 680-740 nm). From (Gamon and Surfus 1999).

1.7. Remote detection of xanthophyll de-epoxidation state and LUE

Violaxanthin and zeaxanthin differ slightly in their reflectance at 531 nm, and their inter-conversion (e.g. under dark-light transition) can be seen as a slight change in leaf reflectance. As a result, dynamic changes in energy dissipation and the xanthophyll cycle are accompanied by a reflectance change in a narrow region of the visible spectrum centered at 531 nm (Gamon et al. 1992); (Stylinski et al. 2002; Stylinski et al. 2000). An appropriate reflectance index to detect these changes in reflectance requires two narrow wavebands: one centered on 531 nm (R_{531}), which is affected by the de-epoxidation of the xanthophyll pigments, and a reference waveband centered at 570 nm (R_{570}), which remains unaffected by the de-epoxidation reaction (Gamon et al. 1992).

This led to the development of the Photochemical Reflectance Index (PRI), defined as (Penuelas et al. 1995):

$$PRI = \frac{R_{531} - R_{570}}{R_{531} + R_{570}}$$

PRI has been found to be strongly correlated with LUE at the leaf scale, small canopy scale and recently at the ecosystem scale (Nichol et al. 2000; Nichol et al. 2002; Rahman et al. 2004).

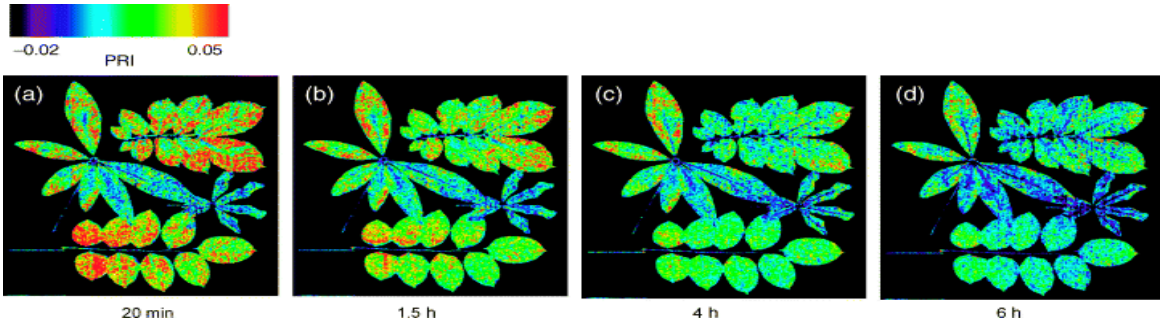

Fig. 9 Changes in photochemical reflectance index (PRI) of leaves of four tropical trees during drying. Leaf reflectance was measured after excising the leaves and allowing evaporation for 20 min, 1.5, 4, and 6 h. The species were Pterocarpus indicus, Ceiba pentandra, Pachira aquatica and Inga cf. sapindoides. From (Rascher et al. 2007).

1.8. Remote sensing of solar-induced fluorescence Fs

Upon absorbing a photon, a chlorophyll molecule jumps to a higher energy state (vibrational level); whatever the energy of the photon absorbed, part of the energy is then dissipated internally, through the decay to the lowest energy level compatible with the vibrational level (corresponding to the energy of a red photon). If the excited chlorophyll molecule then returns to its base state through the emission of a photon (chlorophyll fluorescence), this will have a lower energy (longer wavelength) than the one originally absorbed. Fluorescence is aptly defined as the re-emission of light energy by a pigment molecule at a longer wavelength than the excitation energy.

The emission spectrum of chlorophyll fluorescence is therefore shifted to longer wavelengths than the absorption spectrum of the pigment, within the waveband 650–800 nm with peaks at 690 and 740 nm (Papageorgiou and Govindjee 2004)

.

Since the process competes with photochemistry and heat dissipation, its measurement by RS techniques can be used to gain information about the other two processes.

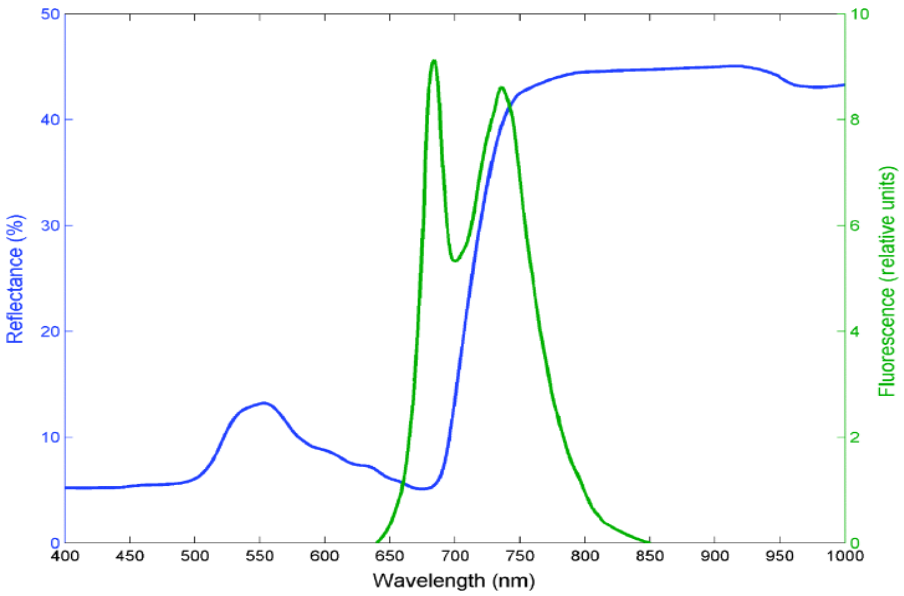

Fig. 10 Characteristic chlorophyll fluorescence emission spectrum (green line) with double-peaks at 685 nm and 740 nm. Superimposed is a characteristic leaf reflectance spectrum (blue line). Fluorescence emission occurs at a longer wavelength than absorption by chlorophyll (Zarco-Tejada et al. 2006).

Photons exiting the leaf because of chlorophyll fluorescence cannot be easily distinguished from those reflected by the leaf. A technique has been developed for their discrimination, the so-called Fraunhofer line in-filling method (Plascyk 1975). The definition is somehow incorrect, as Fraunhofer lines are regions of the solar emission spectrum that are severely depleted as a result of its chemical composition.

The method relies, on the contrary, on specific regions of the spectrum where the light reaching the Earth is strongly depleted because of absorption by oxygen and hydrogen in the atmosphere. The strongest absorption occurs at 760 nm (O_2 -A band), which corresponds to a peak in leaf fluorescence.

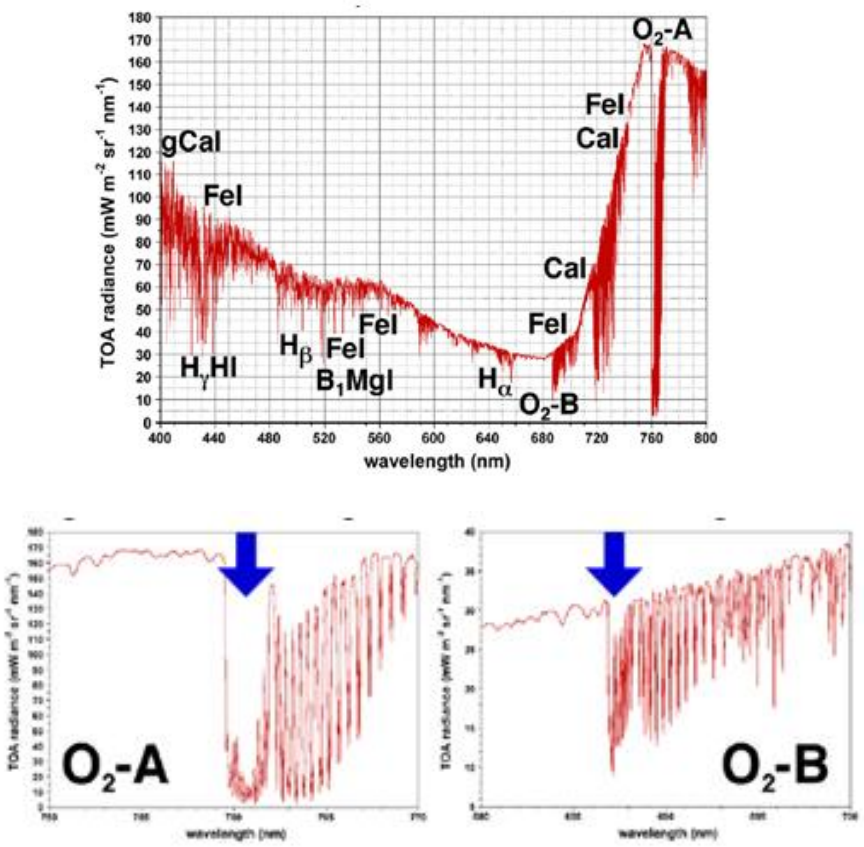

Fig. 11 Location of Fraunhofer lines and absorption bands in the VIS-NIR region, for a typical top-of-atmosphere vegetation radiance spectrum (top panel) and magnified detail of the oxygen absorption bands used for the remote passive detection of chlorophyll fluorescence (bottom panel).

Canopy radiance is also low in these regions, but it is slightly enriched because of the contribution of chlorophyll fluorescence. If we compute leaf reflectance (as

I_{out} / I_{in}), the contribution of fluorescence will show as an apparent peak at 760 nm.

This provides the basis for the remote, passive detection of the chlorophyll fluorescence signal by the “Fraunhofer line in-filling technique”. Canopy radiance outside (c) and inside (d) the Fraunhofer band will be a function of incoming radiation (a and b, respectively) and reflectance (R) but also of the contribution from fluorescence (*f*).

$$c = R \times a + f$$

$$d = R \times b + f$$

True reflectance and fluorescence can be therefore estimated as:

$$R = (c - d) / (a - b)$$

$$f = (a \times d - c \times b) / (a - b)$$

The fluorescence yield estimated by the “Fraunhofer line in-filling technique” using the O_2 -A (at 760 nm) or O_2 -B band (at 680 nm) has been found to be strongly correlated with photosystem II (PSII) light-use efficiency, as estimated by other means on the same leaf (Moya et al. 2004; Perez-Priego et al. 2005)

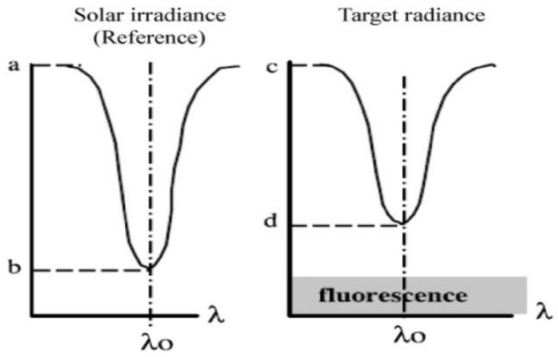

Fig. 12 Rationale of the Fraunhofer line in-filling principle for the remote, passive detection of chlorophyll fluorescence and its discrimination from superimposed leaf reflectance. See text for details.

1.9. Conclusions

Out of the previous review we can draw some general conclusions about the application of remote sensing techniques to ecological research in forestry. Despite the uncertainty connected to remote sensing, this technique has started to be a useful instrument to investigate forest function. Some of the proposed approaches rest on a strong physical and physiological background. The traditional approach, based on broad-band sensors and the simple estimation of LAI from NDVI, although very useful in global studies is not suitable for analyses at local or short-term scale. New methods based on the sensing of the leaf biochemistry and photosynthesis are being developed and applied in pilot studies (PRI, solar-induced fluorescence; N and chlorophyll content) , but still they cannot be applied at global scale for reasons of costs (sensors onboard of airplanes, a new generation of satellites is in developing phase)

Non-destructive monitoring methods, a direct application of RS studies, are increasingly attractive for the determination of stress conditions or nutrient deficiencies not only in research but also in agronomy, horticulture and urban forestry (*proximal remote sensing*).

In this work we will focus on some novel techniques used for the estimation of photosynthetic rates or gross primary productivity from remotely sensed absorbed radiation based on the hyperspectral remote sensing of the changes in the energy partitioning, which is closely coupled to photosynthesis. One of the hyperspectral approaches proposed relies on the remote detection of fluorescence, which is directly related to the efficiency of photosynthesis.

The proximal detection of chlorophyll fluorescence has long been used in ecophysiological studies for the assessment of leaf photochemistry and as an index of photosynthetic processes (Rabinowich and Govindjee 1969; Maxwell and Johnson 2000). Chlorophyll fluorescence measurements generally rely on the application of pulses of saturating light for the estimation of PSII photochemical yield and electron transport rates (Genty et al. 1989). The use of fluorescence measurements under ambient light, however, would be desirable in order to extend the applicability of the technique to passive remote sensing applications. Therefore, we studied in the second chapter of this work the interactions between PSII fluorescence under ambient light conditions and

photochemistry and the resulting link with photosynthetic rates, which could be extended to passive remote sensing applications.

We estimated the fluorescence yield (Φ_f) and the PSII photochemical yield (Φ_{PSII}) under controlled conditions (variable CO_2 and light conditions) using a gas-exchange analyzer (LiCor Li-6400) for measurements of photosynthetic rates, and modulated fluorometer (PAM-2000) for measurements of chlorophyll fluorescence parameters of individual fully expanded mature leaves of two different species: *Arbutus unedo*, a schlerophyllous Mediterranean species, and *Populus euroamericana*, a broad leaf deciduous tree.

In chapter three we present and describe the thorough test of the mathematical model of leaf steady-state fluorescence and photosynthesis developed by Professor Federico Magnani, and referred to as Magnani model (Magnani et al. 2009). It will be discussed first in this chapter the correspondence of modulated and solar-induced fluorescence measurements. A qualitative test of the Magnani model will be presented, based on literature data. And finally, the model will be quantitatively tested against leaf-level data collected with this specific purpose under controlled environmental conditions.

The effects of nitrogen fertilization on fluorescence and heat dissipation and changes in photosynthetic potentials were studied in chapter four, as a suitable test of the predictions of Magnani model. Fluorescence yield (Φ_f) and PSII photochemical yield (Φ_{PSII}) were estimated under controlled conditions (variable CO_2 and light conditions) measuring photosynthetic rates using gas-exchange analyzer (LiCor Li-6400), and chlorophyll fluorescence parameters using modulated fluorometer (PAM-2000) against leaf-level of individual fully expanded mature leaves of *Populus euroamericana* which is a species of interest in forestry. Leaves used for measurements were chosen from those of the maximum or minimum chlorophyll content using the chlorophyll meter (SPAD-502) which is a portable diagnostic tool that measures the greenness or the relative chlorophyll content of leaves. Simultaneous measurements of leaf reflectance were performed using Ocean Optics USB-2000 fibre Optic Spectrometer, and the relationship between the Photochemical Reflectance Index (PRI) and the photochemical yield was tested.

Finally, the fifth chapter focuses on the estimation of the effects of fertilization and nutrient availability on growth, and on the assessment of different RS methods for the determination of leaf nutritional status and biochemical content. These remote sensing techniques could provide useful tools for the assessment of the need for fertilization in plantation forestry, and the analysis of health conditions in natural vegetation. LiCor Li-1800 Spectroradiometer was also used in the analysis, for the measurement of the reflectance and transmittance of the leaves, to be used for the estimation of the photosynthetic pigments content. Several spectral reflectance indices proposed in the literature were tested in order to determine the best one for detecting the changes in leaf photosynthetic pigments content. Two different methods were tested, in particular, for the assessment of leaf chlorophyll, carotenoid and nitrogen content: one is based on the SPAD; a commercial two band spectrometer widely used in field studies, the other is based on the red-edge index position (REIP), a destructive feature of leaf reflectance that is known to be related to leaf chlorophyll content. The study compared these methods with more traditional wet-chemistry techniques (leaf extraction in *N,N*-dimethylformamide, DMF) for the determination of total nitrogen content, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids and the carotenoids/chlorophyll ratio.

The results of the study could prove useful for both basic and applied research, as remote sensing techniques could provide a fast and reliable method to estimate these parameters in the field, for estimating the nutrient requirements of our plants for optimal growth and therefore for the management of cultivation system.

Reference list

Demmig-Adams, B., & Adams, W.W. (2000). Photosynthesis - Harvesting sunlight safely. *Nature, 403*, 371-+

Dewar, R.C. (1996). The correlation between plant growth and intercepted radiation: An interpretation in terms of optimal plant nitrogen content. *Annals of Botany, 78*, 125-136

Gamon, J.A., Field, C.B., Goulden, M.L., Griffin, K.L., Hartley, A.E., Joel, G., Penuelas, J., & Valentini, R. (1995). Relationships between ndvi, canopy structure, and photosynthesis in 3 californian vegetation types. *Ecological Applications, 5*, 28-41

Gamon, J.A., Penuelas, J., & Field, C.B. (1992). A Narrow-Waveband Spectral Index That Tracks Diurnal Changes in Photosynthetic Efficiency. *Remote Sensing of Environment, 41*, 35-44

Gamon, J.A., & Surfus, J.S. (1999). Assessing leaf pigment content and activity with a reflectometer. *New Phytologist, 143*, 105-117

Genty, B., Briantais, J.M., & Baker, N.R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta, 990*, 87-92

Horler, D. N. H., Dockray, M., & Barber, J. (1983). The red-edge of plant leaf reflectance. International *Journal of Remote Sensing,* 4,273-288

Horton, P., Ruban, A.V., & Walters, R.G. (1994). Regulation of light-harvesting in green plants - indication by nonphotochemical quenching of chlorophyll fluorescence. *Plant Physiology, 106*, 415-420

Lacaze, B., & Joffre, R. (1994). Extracting biochemical information from visible and near-infrared reflectance spectroscopy of fresh and dried leaves. *Journal of Plant Physiology, 144*, 277-281

Magnani, F., & Dayyoub, A. (2009). Modelling chlorophyll fluorescence under ambient conditions. *New Phytologist* in preparation

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence. A practical guide, *J. Exp. Bot*., 51 (345), 659-668

Monteith, J. L. (1972). Solar Radiation and Productivity in Tropical Ecosystems. *Journal of Applied Ecology*, 9, No. 3, 747-766

Moya, I., Camenen, L., Evain, S., Goulas, Y., Cerovic, Z.G., Latouche, G., Flexas, J., & Ounis, A. (2004). A new instrument for passive remote sensing. 1. Measurements of sunlight-induced chlorophyll fluorescence. *Remote Sensing of Environment, 91*, 186-197

Myneni, R.B., & Williams, D.L. (1994). On the relationship between fapar and ndvi. *Remote Sensing of Environment, 49*, 200-211

Nichol, C.J., Huemmrich, K.F., Black, T.A., Jarvis, P.G., Walthall, C.L., Grace, J., & Hall, F.G. (2000). Remote sensing of photosynthetic-light-use efficiency of boreal forest. *Agricultural and Forest Meteorology, 101*, 131-142

Nichol, C.J., Lloyd, J., Shibistova, O., Arneth, A., Roser, C., Knohl, A., Matsubara, S., & Grace, J. (2002). Remote sensing of photosynthetic light use efficiency of Siberian boreal forest. *Tellus, 54B*, 677-687

Ollinger, S.V., Richardson, A.D., Martin, M.E., Hollinger, D.Y., Frolking, S.E., Reich, P.B., Plourde, L.C., Katul, G.G., Munger, J.W., Oren, R., Smithb, M.L., U, K.T.P., Bolstad, P.V., Cook, B.D., Day, M.C., Martin, T.A., Monson, R.K., & Schmid, H.P. (2008). Canopy nitrogen, carbon assimilation, and albedo in temperate and boreal forests: Functional relations and potential climate feedbacks. *Proceedings of the National Academy of Sciences of the United States of America, 105*, 19336-19341

Papageorgiou, G.C., & Govindjee (Eds.). (2004). Chlorophyll a Fluorescence. A Signiture of Photosynthesis. Dordrecht: Springer

Penuelas, J., Filella, I., & Gamon, J.A. (1995). Assessment of Photosynthetic Radiation-Use Efficiency with Spectral Reflectance. *New Phytologist, 131*, 291-296

Perez-Priego, O., Zarco-Tejada, P.J., Miller, J.R., Sepulcre-Canto, G., & Fereres, E. (2005). Detection of water stress in orchard trees with a high-resolution spectrometer through chlorophyll fluorescence in-filling of the O-2-A band. *IEEE Transactions in Geoscience and Remote Sensing, 43*, 2860-2869

Plascyk, J. A. (1975). The MK II Fraunhofer line discriminator (FLD-II) for airborne and orbital remote sensing of solar-stimulate luminescence. *Optical Engineering,* 14,339-346

Rabinowich, E., & Govindjee, (1969). Photosynthesis, New York:John Wiley and Sons

Rabinowich, B., Briantais, J. M., & Baker, N. R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochemica et Biophysica Acta,* 990, 87-92

Rahman, A.F., Cordova, V.D., Gamon, J.A., Schmid, H.P., & Sims, D.A. (2004). Potential of MODIS ocean bands for estimating CO2 flux from terrestrial vegetation: a novel approach. *Geophysical Research Letters, 31*

Rascher, U., Nichol, C.J., Small, C., & Hendricks, L. (2007). Monitoring spatio-temporal dynamics of photosynthesis with a portable hyperspectral imaging system. *Photogrammetric Engineering and Remote Sensing, 73*, 45-56

Spurr, S. A., (1960). Photogrammetry and Photo-Interpretation. 2nd ed. *Ronald Press, New York*

Stylinski, C.D., Gamon, J.A., & Oechel, W.C. (2002). Seasonal patterns of reflectance indices, carotenoid pigments and photosynthesis of evergreen chaparral species. *Oecologia, 131*, 366-374

Stylinski, C.D., Oechel, W.C., Gamon, J.A., Tissue, D.T., Miglietta, F., & Raschi, A. (2000). Effects of lifelong CO2 enrichment on carboxylation and light utilization of Quercus pubescens Willd. examined with gas exchange, biochemistry and optical techniques. *Plant Cell and Environment, 23*, 1353-1362

Taiz L., & Zeiger E. (1998). Plant Physiology, 4th Edition-Sinauer Associates, Sunderland

Vina, A., & Gitelson, A.A. (2005). New developments in the remote estimation of the fraction of absorbed photosynthetically active radiation in crops. *Geophysical Research Letters, 32*

Woolley, J.T. (1971). Reflectance and Transmittance of Light by Leaves. *Plant Physiology, 47*, 656-&

Wynne, R.H., & Carter, D.B. (1997). Will remote sensing live up to its promise for forest management? *Journal of Forestry, 95*, 23-26

Zarco-Tejada, P.J., Miller, J.R., Pedros, R., Verhoef, W., & Berger, M. (2006). FluorMODgui V3.0: A graphic user interface for the spectral simulation of leaf and canopy chlorophyll fluorescence. *Computers & Geosciences, 32*, 577-591

Chapter 2

The functional relationship between photosynthesis and ambient chlorophyll fluorescence

2.1 Abstract

The estimation of photosynthetic rates and gross primary productivity from remotely sensed data depends, nowadays, on monitoring variations in absorbed solar radiation and environmental stress conditions. However, more exact methods are now required. One of the most recent hyperspectral approaches proposed relies on the remote detection of ambient chlorophyll fluorescence, which is directly related to the efficiency of photosynthesis.

The prospect of measuring photosynthesis from space is an exciting one. We would be able to track the responses of photosynthesis to climatological variables at several scales as climatic warming and CO_2 fertilization gather pace. Global vegetation models (Cramer et al. 2001) have attempted to capture this response, but unless these models are to be informed by measurements of photosynthesis they remain hypothetical. Now it may be technically possible to achieve these measurements from sensors on board satellites and ground-based flux measurements.

Chlorophyll fluorescence measurements generally rely on the application of pulses of saturating light for the estimation of PSII photochemical yield and electron transport rates. The use of fluorescence measurements under ambient light, however, would be desirable in order to extend the applicability of the technique to passive remote sensing applications.

Although recent advances in quantitative remote sensing have made it possible to measure ambient fluorescence from aerial platforms, background information on the functional significance of ambient fluorescence from leaf-level, detailed

measurements under controlled conditions is still missing. Here we study the interactions between PSII fluorescence and photochemistry and of the resulting link with photosynthetic rates using measurements obtained from gas-exchange analyzer (LiCor Li-6400) and modulated fluorometer (PAM-2000).

Results of the relationship between fluorescence yield and PSII photochemical yield show a segmented pattern, with a positive association under CO_2-limited conditions and a negative linear relationship under light-limited conditions. This observation, which is largely consistent with recent models of ambient fluorescence, could help explain the contrasting reports available in the literature, from studies under contrasting conditions.

Measurements were performed under controlled conditions (variable [CO_2] and light conditions) against leaf-level of individual leaves of *Arbutus unedo*, a schlerophyllous Mediterranean species, and *Populus euroamericana*, a broad leaf deciduous tree. The results show a rather similar response and general pattern in the two different species.

2.2. Introduction

When one photon is intercepted by a chlorophyll molecule in the leaf chloroplast, it can undergo several distinct fates, as the excited singlet chlorophyll molecule can dissipate its energy by four different mechanisms: an electron can be stripped from a donor molecule, initiating electron transport and photochemistry, the primary step of photosynthesis, resulting eventually in ecosystem gross primary production (GPP) and C sequestration. Alternatively, energy can be dissipated as heat, a process up-regulated by any limitations of photosynthetic dark reactions; finally, a small fraction of absorbed energy is re-emitted by chlorophyll at longer wavelengths, a process known as chlorophyll fluorescence (Demmig-Adams and Adams 2000). The formation of chlorophyll triplets, leading to the formation of singlet oxygen and photo-oxidative damage and ultimately cell death, is a rarer event under normal conditions.

Since the three components are intimately related, chlorophyll fluorescence represents a real signature of photosynthesis and could provide useful information for ecological models of the C cycle of terrestrial ecosystems.

Being the main processes involved, photochemistry and thermal energy dissipation are inversely related, and can be (in first principle) estimated the one from the other.

The dissipation of excess energy as heat is known to be the result of the interactions between chlorophyll and leaf xanthophylls in photosystem II (PSII) (Horton et al. 1994).

Xanthophylls are auxiliary pigments, which can be found in three easily inter-convertible forms (zeaxanthin, violaxanthin and the intermediate form antheraxanthin); the de-epoxidation of violaxanthin to zeaxanthin (via antheraxanthin) is a reversible process driven by changes in lumen pH. This typically reflects in diurnal cycles of zeaxanthin content, in response to changes in light and lumen pH, which result in a corresponding pattern in energy dissipation as heat (non-photochemical quenching; Taiz and Zeiger, 1998).

Upon absorbing a photon, a chlorophyll molecule jumps to a higher energy state (vibrational level); enabling it to donate an electron and initiate the transfer of chemical energy needed for photosynthesis. Whatever the energy of the photon absorbed, part of the energy is then dissipated internally, through the decay to the lowest energy level compatible with the vibrational level (corresponding to the energy of a red photon). If the excited chlorophyll molecule then returns to its base state through the emission of a photon (chlorophyll fluorescence), this will have a lower energy (longer wavelength) than the one originally absorbed (Zarco-Tejada et al. 2006). So, because of intervening losses (vibrational relaxation), the photon is re-emitted at a longer wavelength than the one originally absorbed, with two distinct peaks in the R-FR region and that was aptly defined as fluorescence.

The emission spectrum of chlorophyll fluorescence is therefore shifted to longer wavelengths than the absorption spectrum of the pigment, within the waveband 650–800 nm with peaks at 690 and 740 nm (Papageorgiou and Govindjee 2004).

Since the process competes with photochemistry and heat dissipation, its measurement by RS techniques can be used to gain information about the other two processes (Papageorgiou and Govindjee 2004).

Chlorophyll fluorescence has been the subject of a wide body of studies over the last decades to probe the functioning of chemical processes. These studies, however, provide little information of direct use for the analysis of solar-induced fluorescence and its link with photosynthesis. Most studies have applied the so-called pulse-saturated technique, which combines records under ambient conditions (F_t) with the measurement of fluorescence in response to a short (1s) flash of saturating light (F_m^o, F_m'). This makes it possible to eliminate one of the three terms of the energy balance, estimating the remaining two from each other and deduce the electron transport rate and the photochemical quantum yield of PSII (Φ_{PSII}), i.e. the fraction of PSII absorbed light used for photochemistry and electron transport. The latter can be easily estimated from such active measurements as (Genty et al. 1989):

$$\Phi_{PSII} = \frac{\Delta F}{F_m^{'}} = \frac{F_m^{'} - F_t}{F_m^{'}}$$

Photosystem II fluorescence yield (fraction of PSII absorbed light dissipated as fluorescence) can also be estimated from the same set of measurements. In the widely applied modulated fluorometer, fluorescence under ambient conditions (as well as under saturating conditions) is determined from the periodic changes in reflected near-infrared light induced by a dim modulated (0.6 kHz) light, the so-called measurement light.

Since the measurement light is of constant intensity and so weak as not to interfere with PSII status, the fluorescence signal is proportional to fluorescence yield under background light conditions ($F_t \propto \Phi_f$). It is therefore possible to obtain concurrent measurements of fluorescence yield (Φ_f), that is proportional to F_t signal, and PSII photochemical yield (Φ_{PSII}), as assessed through $\Delta F/ F_m$' using the saturating pulse technique, and evaluating their mutual relationship.

Despite its sound theoretical basis, the measurement of apparent quantum yield by the pulse-saturated technique does not lend itself to remote sensing

applications, out of practical and safety considerations, and the use of F_t alone would be highly desirable.

Although robust interpretative models have long been available for the interpretation of pulse-saturation measurements (Genty et al. 1989; Hendrickson et al. 2004), however, they do not apply to the measurement of ambient fluorescence (i.e. F_t alone)

Several studies prove, however, that ambient fluorescence does contain a signal that could be used as a signature of photosynthesis.

Flexas et al. (2002) for example found that measurements of steady-state chlorophyll fluorescence F_t (named F_s in the original study) normalized to dark-adapted F_0 was proportional to net CO_2 assimilation (A) and electron transport rate (ETR), and it was inversely proportional to non-photochemical quenching (NPQ), a widely applied index of heat energy dissipation. For this, they suggest that if it is known just F_t it could be possible to estimate ETR, which could be very useful under a practical perspective. They found also that F_t/F_0 was well correlated with stomatal conductance (g_s); the measurement of chlorophyll fluorescence under ambient light could therefore be a good indicator for the detection of water stress because of the correlation between water stress and stomatal conductance. These findings can be useful for long term monitoring of water stress and other stress conditions by directly monitoring of F_t by passive remote sensing techniques, without the imposition of saturating flashes of light (Flexas et al. 2000; Ounis et al. 2001; Moya et al. 2003).

The temporal variability of the ambient fluorescence signal has also been demonstrated in a recent study on *Pinus sylvistris* by the new MoniPAM modulated fluorometer (Porcar-Castell et al. 2008). Together with changes in the potential (dark-acclimated) and actual PSII apparent quantum yield, substantial fluctuations in the F_t signal were also observed over the course of the day and over the season, although the relationship with photochemical yield was not straightforward.

Other studies have also monitored the time course of fluorescence radiance (as opposed fluorescence yield) under laboratory and field conditions by the

Fraunhofer line in-filling technique (Schmuck et al. 1992; Cerovic et al. 1996; Flexas et al. 1998, 1999), which despite the need for more complex computations could prove more suitable for airborn and satellite remote sensing (Malenovsky et al. 2009)

A number of process-based models have been proposed over the last few years to try and explain the observed variability in solar-induced fluorescence (Rosema et al. 1998; van der Tol et al. 2008), but have not been thoroughly tested. A proper analysis of the response of fluorescence radiance or fluorescence yield to environmental parameters under controlled conditions, and of their relationship with photochemical quantum yield and electron transport is missing so far.

The objective of the present work was therefore to study under controlled conditions (variable CO_2 and irradiance) the relationship between PSII fluorescence and photochemistry, and the resulting link with photosynthetic rates, on individual fully expanded mature leaves of two contrasting species (*Arbutus unedo* and *Populus euroamericana*) so as to provide a sounder basis for future passive remote sensing applications.

2.3. Material and methods

2.3.1. Plant material

Five plants of strawberry trees (*Arbutus unedo* L.) and poplar (*Populus euroamericana*), were grown in pots of 11.3 l capacity containing a mixture of sand and peat (1:1, by volume). The pots were maintained during the experiment period in an open area at the Faculty of Agriculture of the University of Bologna with adequate watering conditions.

Arbutus unedo is an evergreen sclerophyllous tree native to the Mediterranean regions; the species is insect pollinated and can not reach large dimensions. It is drought tolerant and exhibits several mechanisms of drought stress resistance. The effects of drought and excessive radiation on photochemical efficiency, photosynthesis, and carotenoid composition of strawberry trees have been

previously reported (Demmig-Adams et al. 1989; Werner et al. 1999). An increase of zeaxanthin and ascorbate contents and a decrease of chlorophyll, lutein, and β-carotene contents in strawberry tree plants exposed to a combination of severe water deficit, high irradiance, and high temperature has been reported (Munne-Bosch and Penuelas 2004). The response of photosynthetic processes to stress conditions in *A. unedo* seedlings has also been analyzed by Baraldi et al. (2008), who demonstrated a high potential of photoprotection and light dissipation as heat, as a result of the acclimation and overnight retention of zeaxanthin. Also (Levizou et al. 2004) reported a predawn retention of the deepoxidized zeaxanthin as the effect of other micro-environmental conditions (such as high CO_2 concentration) within strawberry tree twigs.

Populus euroamericana is a widely planted hybrid of *P. nigra* and *P. deltoids*, two species of poplar in the cottonwood (*Aegiros*) section of the genus *Populus*. The two species are widely distributed in the northern hemisphere. It is a medium-sized to large deciduous tree, reaching 20-30 m (rarely 40 m) tall, with a trunk up to 1.5 m diameter (Rushforth 1999). The species is dioecious (male and female flowers on different plants), with flowers in catkins and pollination by wind. The response of PSII photochemistry to environmental stress in *P. euroamericana* has also been widely studied (Ridolfi and Dreyer 1997; Guo and Trotter 2004).

Fig. 1 Potted plants of *Arbutus unedo* (to the left) and *Populus euroamericana* (to the right).

2.3.2. Experimental setup and measurements

The experiment was conducted in the laboratory of ecophysiology of the DCA in the Faculty of Agriculture-University of Bologna (Italy). Before each set of measurements, the plant was exposed to high light conditions for one hour in order to acclimate the leaves and activate the photosynthesis process.

Measurements were curried out under controlled conditions on fully expanded mature leaves. The leaf was enclosed inside the broadleaf cuvette of a LiCor Li-6400 gas-exchange analyzer (LI-Cor Inc., Lincoln, NE, USA), which also provided gas exchange measurements, and exposed them to the light of a dichroic halogen lamp. The lamp was connected to an energy stabilizer in order to provide the light intensity needed. Simultaneously chlorophyll fluorescence parameters were measured by pointing the fiberoptics probe of a PAM-2000 modulated fluorometer (Heinz Walz Gmbh, Effeltrich, Germany) at the surface of the leaf through a sealed opening in the leaf cuvette. The setup of the experiment is illustrated in Fig. 2 and 3 .

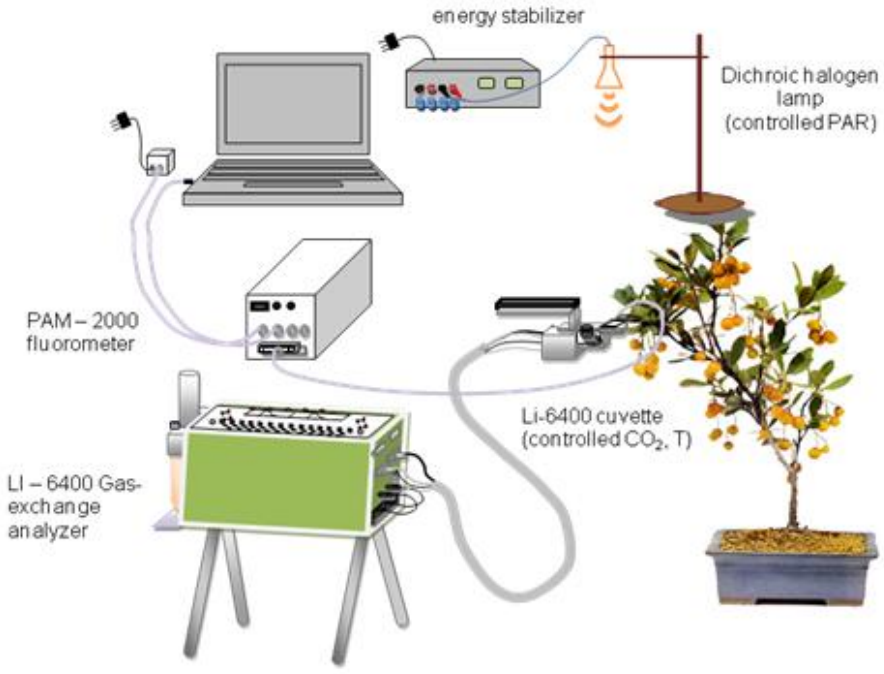

Fig. 2 Scheme of the set-up of the experiment shows leaf of *Arbutus unedo* inside the cuvette of the Li-Cor 6400 (bottom left) exposed to light of halogen lamp connected to an energy stabilizer (top right), and the fiberoptics probe of a PAM-2000 modulated fluorometer (left in the middle) is pointing at the surface of the leaf through a sealed opening in the leaf cuvette.

Fig. 3 The set-up of the experiment: leaf of poplar inside the cuvette of the Li-Cor 6400 (at the bottom) exposed to the light of a halogen lamp connected to an energy stabilizer (to the left), and the fiberoptics probe of a PAM-2000 modulated fluorometer (to the right) is pointing at the surface of the leaf through a sealed opening in the leaf cuvette

Environmental conditions applied on leaves in the LiCor cuvette were varied one at a time, following two different protocols.

In the first protocol, light intensity were changed in steps (1000, 800, 600, 400, 200, 100, 20 μmol m^{-2} s^{-1}) from high to low level, whilst keeping ambient CO_2 inside the cuvette constant (350 ppm). The same steps were then repeated at high (800 ppm) and low (100 ppm) air CO_2 concentration inside the cuvette.

In the second protocol, the air CO_2 concentration inside the LiCor Li-6400 cuvette were changed in steps (350, 200, 50, 350, 550, 800, 1200, 1500 ppm), under moderately low (400 μmol m^{-2} s^{-1}) and high (1000 μmol m^{-2} s^{-1}) light conditions.

The leaf was kept for at least 15 minutes at each level in order to ensure full acclimation. At the end of each step, three successive measurements were made by the LiCor Li-6400 at 2-m intervals, simultaneously with fluorescence measurements by the PAM 2000. Temperature inside the cuvette of the LiCor Li-6400 was maintained at 25 ± 1°C during all the experiment period.

Measurements were repeated with the same experimental setup on seven leaves from separate plants for each species and each protocol.

2.4. Results and discussion

In response to increasing irradiance (PAR, photosynthetically active radiation) at constant air CO_2 concentration there was an increase in the CO_2 assimilation rates of *Arbutus unedo* leaves (Fig. 4A) inside the cuvette of the Li-Cor 6400 until a saturation point where they remained constant. We saw the same pattern with high level of CO_2 concentration giving higher values of photosynthetic rates, and also with low level of CO_2 concentration giving lower values of photosynthetic rates. The same results were seen in the leaves of *Populus euroamericana* (Fig. 4B), where however the relationship between CO_2 assimilation rates and light intensity did not show a clear saturation level under high CO_2 concentrations (350 and 800 ppm).

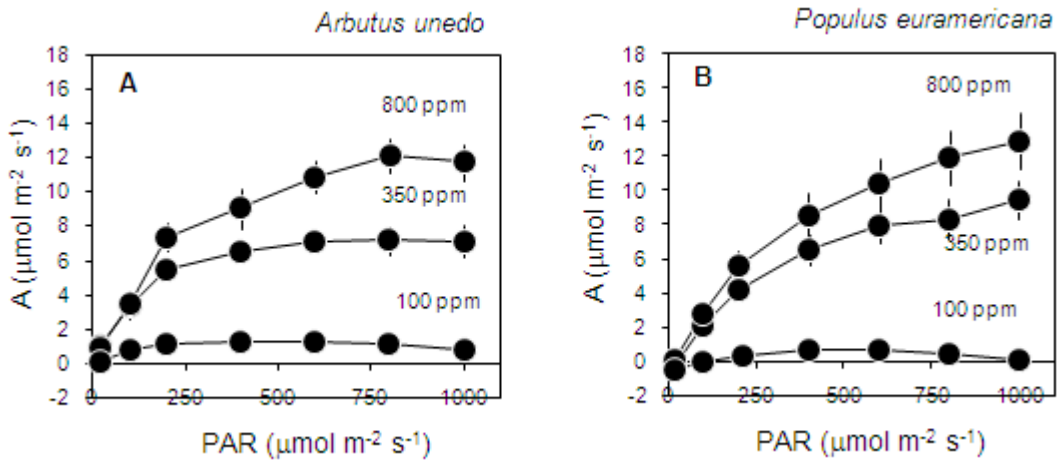

Fig. 4 Response of CO_2 assimilation rate (A) to variation in light intensity (PAR, photosynthetically active radiation) under three different constant levels of CO_2 concentration (800, 350 and 100 ppm) in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7). NB: change figures, lables under the x-axis.

A slight decline in net photosynthesis was observed at high light and low CO_2 concentration, presumably as a result of the slight heating of the leaf and an increase in leaf respiration.

A negative association was found in the relationship between PSII photochemical yield (Φ_{PSII}) and light intensity (PAR) in both *A. unedo* (Fig. 5A) and *P.*

euroamericana leaves (Fig. 5B) at all three CO_2 concentrations.this is the result of increasing PSII centre closure and NPQ buildup, and is in line with what commonly observed (Hendrickson et al. 2005). As a result of increasing saturation of dark reactions, values of photochemical yield at 350 ppm and 800 ppm levels of CO_2 concentration were very close to each other and higher than values at the lowest level of CO_2 (100 ppm).

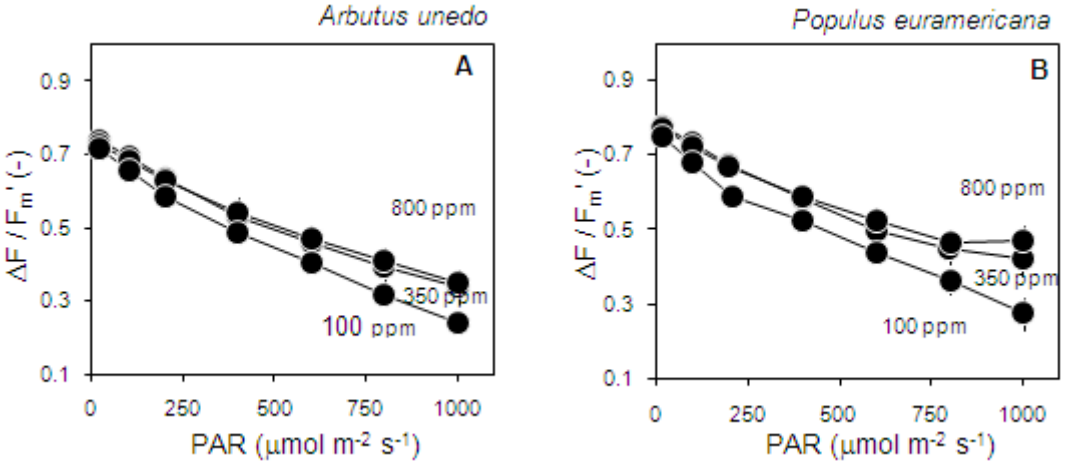

Fig. 5 Response of PSII photochemical yield ($\Delta F/F_m'$) to changes in photosynthetically active radiation (PAR) under three different constant levels of CO_2 concentration (800, 350 and 100 ppm) in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7).

The response of the F_t signal (and therefore of fluorescence yield, Φ_f) to changes in light intensity was different (Fig.6) showing a non-monotonic pattern at all three different levels of CO_2. Fluorescence yield increased with light intensity until a certain level (100 μmol m^{-2} s^{-1} for 100 ppm CO_2 concentration; 200 μmol m^{-2} s^{-1} for 350 and 800 ppm CO_2 concentrations), then it decreased to levels similar or even lower than observed under dim light. At the highest levels of irradiance, however, fluorescence yields appeared to stabilize or even slightly increase. Highest fluorescence yields were observed under 800 ppm CO_2 concentration, and the lowest ones under 100 ppm CO_2 concentration, although the difference was only apparent under high light conditions. Similar relationships were observed in *A. unedo* (Fig. 6A) and in *P. euroamericana* leaves (Fig. 6B), but with lower values of fluorescence yield under the different CO_2 concentrations in the

latter species. Such a curvilinear response of fluorescence yield to increasing irradiance had already been observed by Rosema et al. (1998) using a laser-induced fluorometer. Likewise, a similar pattern of F_t (normalized by dark-acclimated F_o in order to remove any effects of leaf absorption or pre-existing stress) in response to increasing irradiance had already been reported by Flexas (2002), who also noted a substantial decline in F_t at constant light as a result of stomatal closure. The effects of stomatal limitations of photosynthesis, mediated by a decline in CO_2 concentration at carboxylation sites, should be similar to those of a reduction in air CO_2 concentration, as applied in the present study.

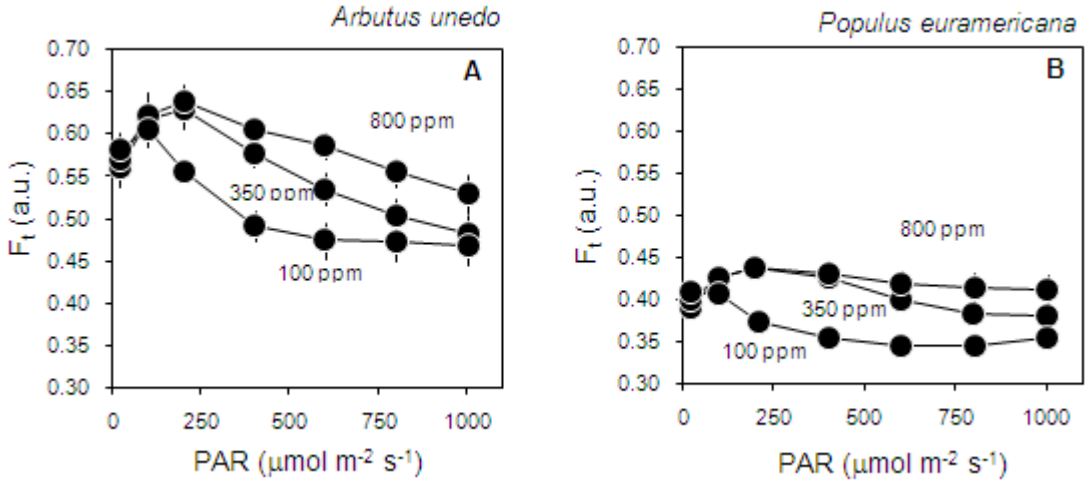

Fig. 6 Response of fluorescence yield (Φ_f) to changes in irradiance (PAR, photosynthetically active radiation) (µmol m^{-2} s^{-1}) under three different constant levels of CO_2 concentration (800, 350 and 100 ppm) in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7).

When considering the relationship between PSII fluorescence yield ($\Phi_f \propto F_t$) and photochemical yield ($\Phi_{PSII} \propto \Delta F/F_m'$) in response to changes in irradiance under three constant CO_2 concentrations (Fig. 7), a non-monotonic relationship is clearly apparent. That when photochemical yield (electron transport yield) increases, fluorescence yield under the three different concentrations of CO_2 increase in parallel until a saturation point, then they collapse to a single point. So, we have a combined variation with a negative association between the two yields under light-limited conditions (in the morning and late afternoon and in

cloudy days for example), and a positive association on the contrary under conditions limited by stomatal closure and CO_2 availability (in the middle of the day and in stress conditions for example). The variable slope of the positive correlation (CO_2 limited conditions) is presumably related to different temperatures. It was interesting the very close resemblance in this relationship between the two species *Arbutus unedo* (Fig. 7A) and *Populus euroamericana* (Fig. 7B) with difference in values of fluorescence yield, which was higher for *Arbutus unedo* than the ones of *Populus euroamericana*.

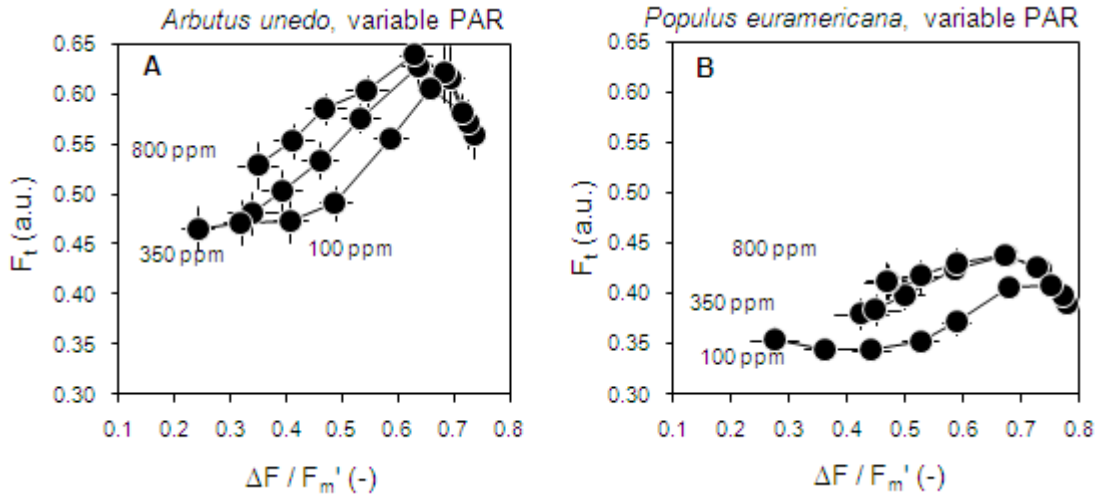

Fig. 7 Relationship between fluorescence yield ($\Phi_f \propto F_t$) and PSII photochemical yield ($\Phi_{PSII} \propto \Delta F/F_m'$) in response to changes in irradiance (PAR) under three constant CO_2 concentrations in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7).

The bi-phasic relationship of F_t with irradiance and apparent quantum yield could help explain the contrasting results reported in the literature. Liu et al. 2005), for example, observed a negative association between fluorescence yield and ΔF/Fm' when the latter ranged between 0.83 and 0.77 (light-limited conditions). A negative relationship between quantum yield and fluorescence yield had also been observed by Rosema et al. (1998) early in the morning, at low light, but a strong hysteresis was apparent in noon data under high radiation loads.
A bi-phasic response of fluorescence yield to light intensity has also been proposed by van der Tol et al. (2008), based on a semi-empirical model of energy

partitioning in PSII. In the model, the response pattern was explained by the different impact of light and dark reactions on fluorescence yield: under light-limiting conditions (low light), NPQ and energy dissipation as heat are supposed not to be entrained, so that fluorescence would only compete with photochemistry and a negative association should be expected between Φ_f and Φ_{PSII}. Under high-light conditions, on the contrary, electron transport would be back-regulated by CO_2 availability and carboxylation rates through the build-up of thylakoid lumen pH and the development of NPQ, which would increase in parallel with excess light; under such conditions, energy dissipation as heat would predominate as a quencher, reducing photochemistry and fluorescence to the same rate. Fluorescence yield should therefore decline with increasing irradiance, and a positive association should be expected between Φ_f and Φ_{PSII}. The slope of the relationship at high irradiance observed in the current study, however, appears to be much steeper than predicted by the van der Tol et al. (2008) model, casting some doubts on its assumptions.

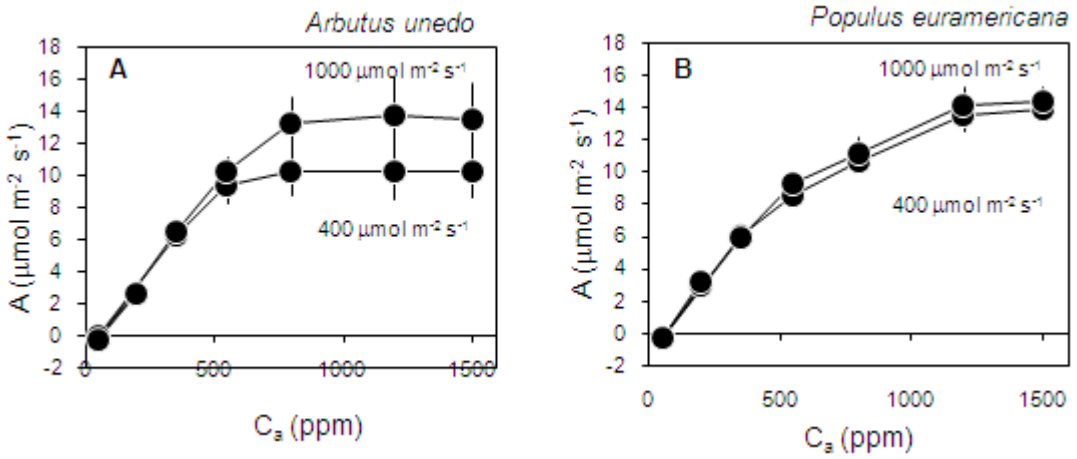

Fig. 8 Response of CO_2 assimilation rate to variation in air CO_2 concentration (C_a) under two different light intensities (PAR) in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7). NB: change figures, labels under the x-axis

An additional test of our understanding of the relationship between fluorescence and photochemical yields comes from measurements under variable CO_2.

The response of CO_2 assimilation rate to variation in CO_2 concentration under two different light intensities (400 and 1000 µmol m^{-2} s^{-1}) is presented in Fig. 8. Photosynthetic rates increased with CO_2 concentration in *A. unedo* (Fig. 8A) until a saturation point (C_a ≈ 800 ppm in). At low CO_2 concentration, photosynthetic rates were limited by electron transport rates alone and were therefore almost identical under different light intensities. An identical pattern was observed in *P. euroamericana* (Fig. 8B), where saturation was reached at a slightly higher CO_2 concentration.

In contrast with net photosynthesis, PSII photochemical yield (Φ_{PSII}) increased only slightly with CO_2 concentration under the two different light intensities in both species (Fig. 9), as photorespiration increasingly complemented carboxylation as an electron sink under low CO_2. Values of PSII photochemical yield under 400 µmol m^{-2} s^{-1} light intensity were higher than those at 1000 µmol m^{-2} s^{-1}, and they rised in parallel with increasing CO_2.

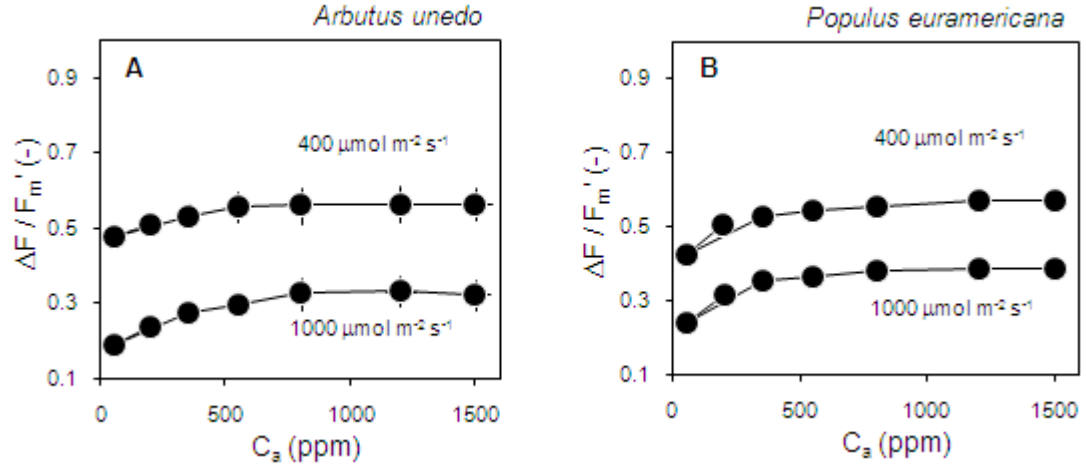

Fig. 9 Response of PSII photochemical yield ($\Phi_{PSII} \propto \Delta F/F_m'$) to changes in CO_2 concentration (C_a) under two different light intensities in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7).

At low CO_2 concentration we noticed that values of fluorescence yield (Φ_f) under the two light intensities were almost equal in both species (Fig. 10A and B). With increasing CO_2 concentrations, fluorescence yield raised under both light intensities until reaching a saturation point, before declining slightly at higher CO_2

concentrations (apart from *P. euroamericana* at 1000 µmol m^{-2} s^{-1}). Fluorescence yield values under a light intensity of 400 µmol m^{-2} s^{-1} were higher than those at 1000 µmol m^{-2} s^{-1}.

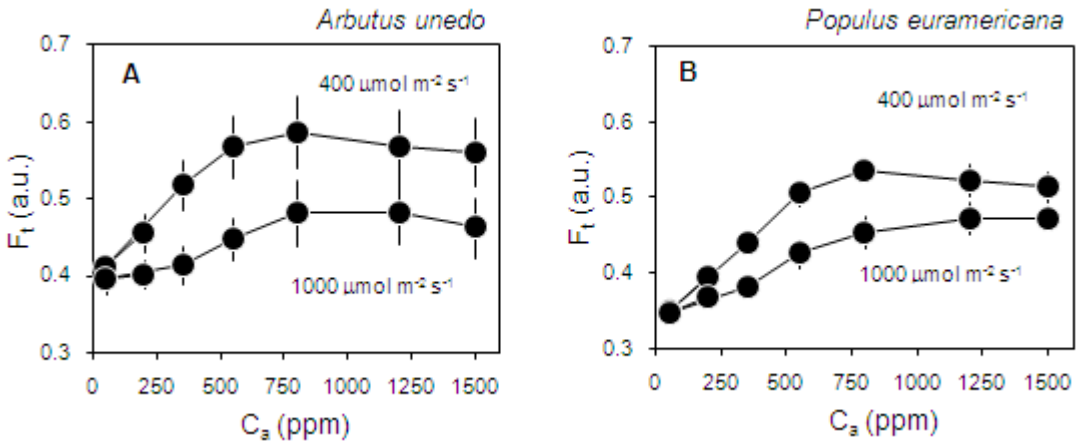

Fig. 10 Response of fluorescence yield ($\Phi_f \propto F_t$) to changes in CO_2 concentration (C_a) under two different light intensities in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7).

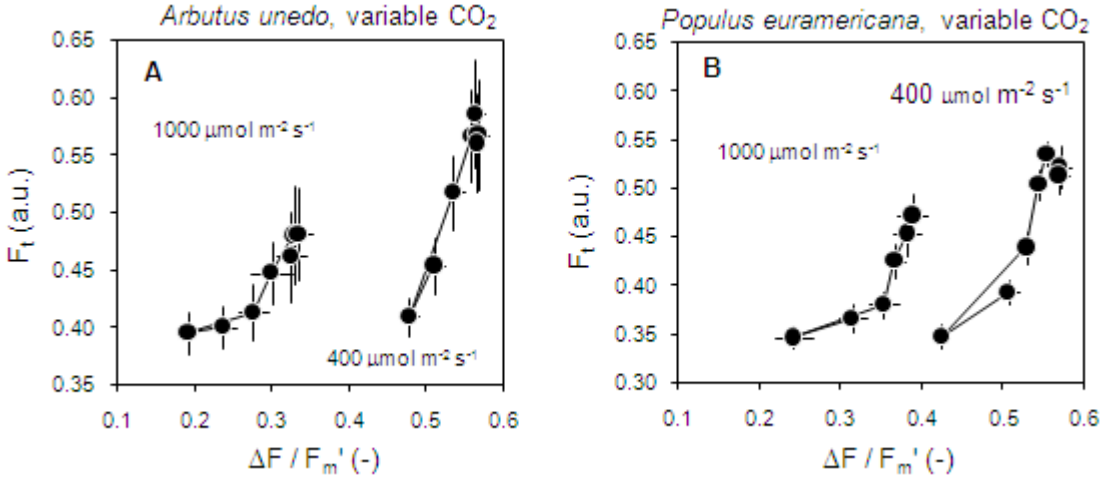

Fig. 11 Relationship between fluorescence yield ($\Phi_f \propto F_t$) and PSII photochemical yield ($\Phi_{PSII} \propto \Delta F/F_m'$) in response to changes in CO_2 concentration under two different light intensities (PAR) in *Arbutus unedo* (A) and *Populus euroamericana* (B) leaves. Mean ± SE (n=7).

In contrast with what observed in response to variable light, a non-monotonic relationship was observed in the relationship between fluorescence yield (Φ_f) and PSII photochemical yield (Φ_{PSII}) in response to changes in CO_2 concentrations under two different light intensities (Fig. 11). In both species the initial value of the fluorescence yield was not affected by light levels, but the final values were much higher under 400 PAR µmol m^{-2} s^{-1} than that under 1000 µmol m^{-2} s^{-1} PAR.

Although not specifically focused on the effects of air CO2 concentrations, consideration of some other studies can help put our results in a broader perspective. Two of them will be discussed, in particular, which have considered the effects of water availability. In first approximation, the reduction in stomatal conductance and CO2 concentration at carboxylation sites brought about by drought can be assimilated in their effects to the decrease in air CO2 applied in the present study. In good agreement with our results, a positive relationship was observed in *Olea europea* by Perez-Priego et al. (2005) between PSII quantum yield and F_t in response to increasing levels of water stress under high light conditions. A similar decline in Ft at midday under drought conditions was observed in *Vitis vinifera* plants by Flexas et al. (1999); this resulted in a positive linear association between Ft and electron transport rates under saturating light (Flexas et al. 2002), implying a parallel positive relationship with PSII quantum yield similar to what reported here. A positive linear correlation between Φ_{PSII} and Φ_f in response to variable CO_2 concentrations is also consistent with the model recently presented by van der Tol et al. 2008), which also predicts, for any given value of PSII quantum yield, a higher fluorescence yield under conditions of strong irradiance, as observed in the current study.

2.5. Conclusions

The study represents the first analysis under controlled conditions of the response to individual environmental factors (PAR, CO_2) of PSII fluorescence yield, and of its relationship with photochemical yield. The response observed in two different species, a broadleaf sclerophyllous tree from the Mediterranean (*A. unedo*), and a broadleaf deciduous tree *(P. euroamericana*), is qualitatively consistent, despite clear differences in photosynthetic rates. There are some

differences between the two species, but the general pattern is the same in response to both variable PAR and variable CO_2.

Based on the results presented, it would appear that ambient fluorescence could provide a useful tool for testing photosynthetic processes from a distance.

The new understanding of ambient fluorescence opens novel perspectives for the airborne and satellite remote sensing of photosynthetic processes.Problems remain however in its interpretation, despite new models being proposed.

The experimental test demonstrates a consistent pattern of co-variation between fluorescence and photochemical yield. However, the relationship differs depending on whether photosynthesis is limited by light (negative association) or CO_2 (positive association). This would appear to explain the contrasting patterns presented in past studies, with both positive and negative associations being reported. Recent models seem to capture some of the key features of the observed pattern, but other important characteristics are clearly not fully explained. Additional modelling efforts are required if the full potential of the ambient fluorescence signal is to be brought to fruition.

Reference list

Baraldi, R., Canaccini, F., Cortes, S., Magnani, F., Rapparini, F., Zamboni, A., & Raddi, S. (2008). Role of xanthophyll cycle-mediated photoprotection in Arbutus unedo plants exposed to water stress during the Mediterranean summer. *Photosynthetica, 46*, 378-386

Cerovic, Z.G., Goulas, Y., Gorbunov, M., Briantais, J.M., Camenen, L., & Moya, I. (1996). Fluorosensing of water stress in plants: Diurnal changes of the mean lifetime and yield of chlorophyll fluorescence, measured simultaneously and at distance with a tau-LIDAR and a modified PAM-fluorimeter, in maize, sugar beet, and Kalanchoe. *Remote Sensing of Environment, 58*, 311-321

Cramer, W., Bondeau, A., Woodward, F.I., Prentice, I.C., Betts, R.A., Brovkin, V., Cox, P.M., Fisher, V., Foley, J.A., Friend, A.D., Kucharik, C., Lomas, M.R., Ramankutty, N., Sitch, S., Smith, B., White, A., & Young-Molling, C. (2001). Global response of terrestrial ecosystem structure and function to CO2 and climate change: results from six dynamic global vegetation models. *Global Change Biology, 7*, 357-373

Demmig-Adams, B., & Adams, W.W. (2000). Photosynthesis - Harvesting sunlight safely. *Nature, 403*, 371-+

Demmigadams, B., Adams, W.W., Winter, K., Meyer, A., Schreiber, U., Pereira, J.S., Kruger, A., Czygan, F.C., & Lange, O.L. (1989). Photochemical efficiency of photosystem-ii, photon yield of o-2 evolution, photosynthetic capacity, and carotenoid composition during the midday depression of net co2 uptake in arbutus-unedo growing in portugal. *Planta, 177*, 377-387

Flexas, J., Escalona, J.M., & Medrano, H. (1999). Water stress induces different levels of photosynthesis and electron transport rate regulation in grapevines. *Plant Cell and Environment, 22*, 39-48

Flexas, J., Briantais, J.M., Cerovic, Z., Medrano, H., & Moya, I. (2000). Steady-state and maximum chlorophyll fluorescence responses to water stress in grapevine leaves: A new remote sensing system. *Remote Sensing of Environment, 73*, 283-297

Flexas, J., Escalona, J.M., Evain, S., Gulias, J., Moya, I., Osmond, C.B., & Medrano, H. (2002). Steady-state chlorophyll fluorescence (Fs) measurements as a tool to follow variations of net CO2 assimilation and stomatal conductance during water-stress in C-3 plants. *Physiologia Plantarum, 114*, 231-240

Genty, B., Briantais, J.M., & Baker, N.R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta, 990*, 87-92

Guo, J.M., & Trotter, C.M. (2004). Estimating photosynthetic light-use efficiency using the photochemical reflectance index: variations among species. *Functional Plant Biology, 31*, 255-265

Hendrickson, L., Furbank, R.T., & Chow, W.S. (2004). A simple alternative approach to assessing the fate of absorbed light energy using chlorophyll fluorescence. *Photosynthesis Research, 82*, 73-81

Hendrickson, L., Forster, B., Pogson, B.J., & Chow, W.S. (2005). A simple chlorophyll fluorescence parameter that correlates with the rate coefficient of photoinactivation of Photosystem II. *Photosynthesis Research, 84*, 43-49

Horton, P., Ruban, A.V., & Walters, R.G. (1994). Regulation of light-harvesting in green plants - indication by nonphotochemical quenching of chlorophyll fluorescence. *Plant Physiology, 106*, 415-420

Levizou, E., Petropoulou, Y., & Manetas, Y. (2004). Carotenoid composition of peridermal twigs does not fully conform to a shade acclimation hypothesis. *Photosynthetica, 42*, 591-596

Liu, L.Y., Zhang, Y.J., Wang, J.H., & Zhao, C.J. (2005). Detecting solar-induced chlorophyll fluorescence from field radiance spectra based on the Fraunhofer line principle. *IEEE Transactions in Geoscience and Remote Sensing, 43*, 827-832

Malenovsky, Z., Mishra, K.B., Zemek, F., Rascher, U., & Nedbal, L. (2009). Scientific and technical challenges in remote sensing of plant canopy reflectance and fluorescence. *Journal of Experimental Botany, 60*, 2987-3004

Moya, I., Cartelat, A., Cerovic, Z.G., Ducruet, J.M., Evain, S., Flexas, J., Goulas, Y., Louis, J., Meyer, S., Moise, N., & Ounis, A. (2003). Possible approaches to remote sensing of photosynthetic activity. *IEEE Transactions in Geoscience and Remote Sensing*, 588 -590

Munne-Bosch, S., & Penuelas, J. (2004). Drought-induced oxidative stress in strawberry tree (Arbutus unedo L.) growing in Mediterranean field conditions. *Plant Science, 166*, 1105-1110

Ounis, A., Evain, S., Flexas, J., Tosti, S., & Moya, I. (2001). Adaptation of a PAM-fluorometer for remote sensing of chlorophyll fluorescence. *Photosynthesis Research, 68*, 113-120

Papageorgiou and Govindjee (2004). *Chlorophyll a Fluorescence. A Signature of Photosynthesis*. Dordrecht: Springer

Perez-Priego, O., Zarco-Tejada, P.J., Miller, J.R., Sepulcre-Canto, G., & Fereres, E. (2005). Detection of water stress in orchard trees with a high-resolution spectrometer through chlorophyll fluorescence in-filling of the O-2-A band. *IEEE Transactions in Geoscience and Remote Sensing, 43*, 2860-2869

Porcar-Castell, A., Pfundel, E., Korhonen, J.F.J., & Juurola, E. (2008). A new monitoring PAM fluorometer (MONI-PAM) to study the short- and long-term acclimation of photosystem II in field conditions. *Photosynthesis Research, 96*, 173-179

Ridolfi, M., & Dreyer, E. (1997). Responses to water stress in an ABA-unresponsive hybrid poplar (Populus koreana x trichocarpa cv Peace) .3. Consequences for photosynthetic carbon assimilation. *New Phytologist, 135*, 31-40

Rosema, A., Snel, J.F.H., Zahn, H., Buurmeijer, W.F., & Van Hove, L.W.A. (1998). The relation between laser-induced chlorophyll fluorescence and photosynthesis. *Remote Sensing of Environment, 65*, 143-154

Rushforth, K., (1999). Trees of Britain and Europe. Harper Collins, New York

Schmuck, G., Moya, I., Pedrini, A., Vanderlinde, D., Lichtenthaler, H.K., Stober, F., Schindler, C., & Goulas, Y. (1992). Chlorophyll fluorescence lifetime determination of water-stressed c3-plant and c4-plant. *Radiation and Environmental Biophysics, 31*, 141-151

Taiz, L., & Zeiger, E. (1998). Plant Physiology. 4th EditionSinauer Associates, *Sunderland.*

van der Tol, C., Verhoef, W., & Rosema, A. (2008). A model for chlorophyll fluorescence and photosynthesis at leaf scale. *Agricultural and Forest Meteorology, 149*, 96-105

Werner, C., Correia, O., & Beyschlag, W. (1999). Two different strategies of Mediterranean macchia plants to avoid photoinhibitory damage by excessive radiation levels during summer drought. *Acta Oecologica-International Journal of Ecology, 20*, 15-23

Zarco-Tejada, P.J., Miller, J.R., Pedros, R., Verhoef, W., & Berger, M. (2006). FluorMODgui V3.0: A graphic user interface for the spectral simulation of leaf and canopy chlorophyll fluorescence. *Computers & Geosciences, 32*, 577-591

Chapter 3

Modelling the response of steady-state chlorophyll fluorescence to environmental factors: model validation in two contrasting species

3.1 Introduction

Upon absorbing a photon, a chlorophyll molecule jumps to a higher energy state; whatever the energy of the photon absorbed, part of the energy is then dissipated internally, through the decay to the lowest energy level available in the excited vibrational state (corresponding to the energy of a red photon). If the excited chlorophyll molecule then returns to its base state through the emission of a photon, this will have a lower energy (longer wavelength) than the one originally absorbed. The process, known as fluorescence, is aptly defined as the re-emission of light energy by a pigment molecule at a longer wavelength than the excitation energy (Baker 2008). Since the process competes with photochemistry and heat dissipation its measurement can be used to gain information about the other two processes.

The measurement of chlorophyll fluorescence from a distance has been proposed over the last few years as a useful tool to investigate vegetation function and productivity from airborne and satellite platforms (Moya *et al.* 2003; Grace *et al.* 2007). Although the technical feasibility of such measurements has been demonstrated (Guanter *et al.* 2007; Joiner *et al.* 2011), the interpretation of the resulting data is not straightforward. Chlorophyll fluorescence measurements have long been used in ecophysiological studies for the assessment of leaf photochemistry and as an index of photosynthetic processes (Rabinowich and Govindjee 1969; Maxwell and Johnson 2000). Chlorophyll fluorescence

measurements, however, generally rely on the application of pulses of saturating light for the estimation of PSII photochemical yield and electron transport rates; although several sound models have been developed over the years for the interpretation of the signal obtained with such a manipulative approach (Genty *et al.* 1989; Kramer *et al.* 2004), however, they cannot be used for the analysis of the steady-state fluorescence signal under ambient light conditions. The use of fluorescence measurements under ambient light, however, would be desirable in order to extend the applicability of the technique to passive remote sensing applications, making the development of novel suitable models a priority in research. A few models have indeed been proposed over the last few years for the interpretation of ambient fluorescence, but they are admittedly only applicable to low-light, unstressed conditions (Rosema *et al.* 1998) or rely on empirical and not fully warranted assumptions about co-limitation of fluorescence and photochemical processes, that are not consistent with current knowledge of energy partitioning (van der Tol *et al.* 2008).

A novel model has been recently developed in our research group (Magnani, unpublished), which tries to overcome these limitations. In the present chapter, the model will be briefly presented and tested against detailed measurements of photochemical processes and fluorescence in two contrasting species under controlled environmental conditions.

3.2 Modelling ambient fluorescence from photosystem II

3.2.1 Model assumptions

We consider a population of N_{PSII} photosystem II (PSII) units, where each PSII has a given amount of chlorophyll *a* and *b* molecules (*Chla* and *Chlb*, respectively, with units of mol mol^{-1}), as well as associated quinone and plastoquinone pools (*Q*, mol mol^{-1}). Since we are interested only in short-term changes in leaf function, apart from xanthophyll de-epoxidation state all other stoichiometries are assumed constant for a given leaf, but potentially different among leaves. The quinone and plastoquinone pools are treated together, since the analysis of the redox state of the different quinone pools falls outside the

scope of the present study. Consequently, we represent the different forms as a single pool of quinone-equivalent molecules, capable of accepting one electron each.

The formation of chlorophyll triplet states is considered part of the constitutive heat dissipation component. Moreover, photoinhibitory NPQ is not yet included in the model. Finally, the state-transition form of NPQ, which is much smaller than the pH-dependent form (Krause *et al.* 1991), is not considered here for simplicity reasons.

Finally, the system of N_{PSII} units is assumed to follow a lake-type organisation model (Kitajima *et al.* 1975; Dau 1994; Bernhardt *et al.* 1999), whereby excitons can move freely between PSII units. The probability of being in an excited state is therefore the same for all chlorophyll *a* molecules in the PSII (including the reaction centre). Although PSII units are known to be only partly interconnected in higher plants (Lazar 1999), the lake model has been demonstrated to yield almost indistinguishable results (Kramer *et al.* 2004).

3.2.2 Processes

In the proposed model, pigment molecules and quinone-equivalents shift from a base (*OFF*) to an excited (*ON*) state after accepting a photon, an exciton or an electron, returning to the OFF state after losing it. Excitation transfer between *Chl b* and *Chl a* is known to be unidirectional and to occur in the order of picoseconds (Dau 1994; Amerongen *et al.* 2003). As a result, at the time scale considered, chlorophyll *b* molecules are assumed to operate only in light absorption and excitons are assumed to be always located on Chla (i.e. $Chlb^{OFF} = Chlb$) (Whitmarsh *et al.* 2002).

From excited chlorophyll *a* molecules, excitons can follow different paths (Fig. 1): they can be re-emitted as fluorescence, lost as constitutive heat, dissipated by pH-dependent heat dissipation processes (non-photochemical quenching) or captured by an oxidized quinone-equivalent (Kitajima *et al.* 1975). Finally, the reduced quinone-equivalents can be re-oxidized by the downstream electron transport, returning to the *OFF* state.

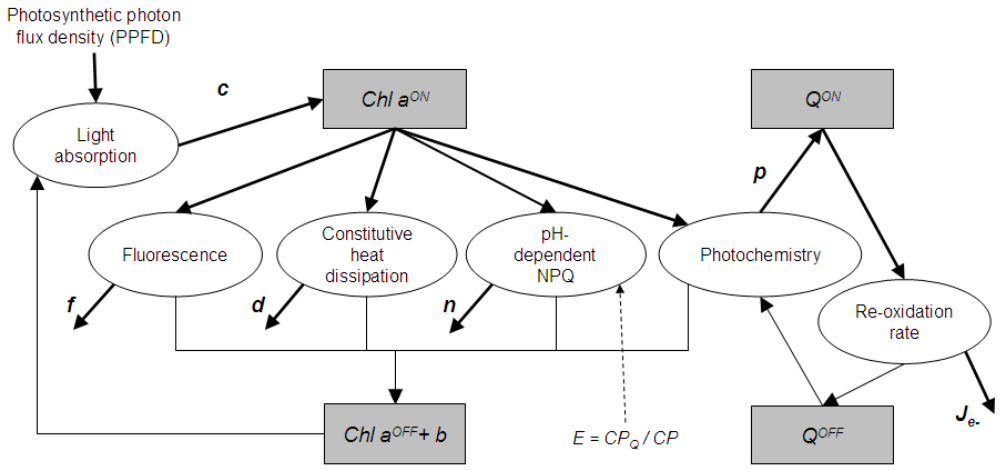

Fig. 1 Scheme of light energy capture and dissipation in photosystem II. Rectangular boxes represent density of a substance in an energy state, circles represent processes. The flow of energy is represented by thick lines, state transitions by continuous thin lines; dashed lines represent an influence. $Chla^{ON}$ and $Chla^{OFF}$ represent the density of chlorophyll a molecules carrying an exciton or in the ground state, respectively. Q^{ON} and Q^{OFF} are the density of quinone-equivalents in a reduced and oxidized state, respectively. E is the fraction of core antenna complexes in a quenched state. f, d, n, p and J_{e-} are the rate of fluorescence, constitutive heat dissipation, pH-dependent non-photochemical heat dissipation, reduction of the quinone-equivalent pool (photochemistry) and electron transport, respectively. From Porcar-Castell et al. (2006, modified).

3.2.3 Dynamic equations

Under steady-state conditions, the rate of light capture (c) must equal the combined rate of energy dissipation by photochemistry (p), fluorescence (f) and heat, either constitutive (d) or energy-regulated (n):

$$c = d + f + n + p \quad (1)$$

All rates are expressed in units of mol m^{-2} s^{-1}.

The rate of constitutive heat dissipation (*d*) and fluorescence (*f*) are linearly proportional to the number of excitons per unit area:

$$d = k_d \cdot N_{PSII} \cdot Chla^{ON} \quad (2)$$

$$f = k_f \cdot N_{PSII} \cdot Chla^{ON} \quad (3)$$

where $Chla^{ON}$ (mol mol^{-1}) is the number of excited chlorophyll a molecules per reaction centre and k_d (s^{-1}) and k_f (s^{-1}) are the rate constants for constitutive heat dissipation and fluorescence, respectively. The rate of energy-dependent heat dissipation (*n*) has been proposed to be a function of lumen pH and xanthophyll de-epoxidation (Gilmore *et al.* 1998). Its detailed representation, however, is beyond the purposes of the present model.

Photochemistry is only initiated when an exciton resides in the reaction centre P680; according to the shallow lake model and the assumption of equal exciton distribution among all Chl *a* molecules (van Grondelle 1985), this will occur with a probability $Chla_{RC}^{ON} = Chla^{ON} / Chla$ (Lavergne *et al.* 1995; Zhu *et al.* 2005).

The efficiency of photochemistry also varies according to the redox state of the quinone-equivalent pool: when the pool is totally reduced, the rate of photochemistry is zero, whilst it is at its maximum when the plastoquinone pool is totally oxidized. Using a Stern-Volmer type approach, the rate of photochemistry (*p*) will be proportional to the oxidized fraction of the quinone-equivalent pool ($= Q^{OFF} / Q$), analogous to the commonly referred to fraction of open reaction centres, q_L (as defined by Kramer et al. 2004):

$$p = k_p \cdot \frac{Q^{OFF}}{Q} \cdot Chla_{RC}^{ON} \cdot N_{PSII} = k_p \cdot \frac{Q^{OFF}}{Q} \cdot \frac{Chla^{ON}}{Chla} \cdot N_{PSII} \quad (4)$$

where k_p (s^{-1}) is the rate constant associated with photochemical processes. The dependence of *p* upon *Chla* has been confirmed by measurements of the net rates of charge separation for PSII of different antenna size: the higher the number of chlorophyll *a* molecules associated with a PSII reaction centre, the lower rate of primary charge separation (Holzwarth *et al.* 1985; Schatz *et al.* 1987).

The rate of electron transport to PSI (J_{e^-} ; mol m^{-2} s^{-1}) determines the rate at which the quinone-equivalent pool is re-oxidized, and will be proportional to the number of quinone equivalents in a reduced state per PSII (Q^{ON}; mol mol^{-1}). Under steady-state conditions, this must be equal to the rate of photochemistry (*p*), which determines the rate of reduction of the quinone-equivalent pool:

$$J_{e^-} = \gamma \cdot Q^{ON} \cdot N_{PSII} = p \qquad (5)$$

where γ (s^{-1}) is the rate constant for the re-oxidation of the quinone-equivalent pool.

3.2.4 Modelling chlorophyll fluorescence

Under steady-state conditions, the rate of exciton transfer to PSII (a function of chlorophyll excitation and quinone reduction state) must be balanced by charge separation and electron transport initiation (itself a function of quinone reduction state). By combining Eq. 4 and 5, electron transport rate can be expressed as a function of the number of excited chlorophyll *a* molecules per PSII ($Chla^{ON}$) as (see Appendix 1 for a detailed description of all the steps involved in the following equations):

$$J_{e-} = N_{PSII} \cdot \frac{k_p \cdot \frac{Chla^{ON}}{Chla} \cdot \gamma \cdot Q}{\gamma \cdot Q + k_p \cdot \frac{Chla^{ON}}{Chla}} \quad (6)$$

Alternatively, the number of excited chlorophyll *a* molecules per PSII can be expressed as a function of electron transport rates:

$$Chla^{ON} = \frac{Chla}{k_p} \cdot \frac{\gamma \cdot Q \cdot J_{e-}}{\gamma \cdot N_{PSII} \cdot Q - J_{e-}} \quad (7)$$

By combining Eq. 10 above with Eq. 3, which represents the dependence of fluorescence rates upon $Chla^{ON}$, fluorescence *f* can be finally expressed as a function of electron transport rates:

$$f = \frac{k_f}{k_p} \cdot Chla \cdot \frac{N_{PSII} \cdot \gamma \cdot Q \cdot J_{e-}}{N_{PSII} \cdot \gamma \cdot Q - J_{e-}} \quad (8)$$

where $N_{PSII} \cdot \gamma \cdot Q$ is the electron transport rate that could be expected if all the quinone-equivalent pool were in a reduced state (i.e. if all reaction centres were closed).

Fluorescence is known to be strongly affected by leaf chlorophyll content. This is particularly true for the fluorescence peak at 760 nm, where the fluorescence signal is not affected by re-absorption by leaf chlorophyll itself. As a result, solar-induced fluorescence and the ratio of fluorescence at 730 vs 680 nm have been proposed as an index of leaf chlorophyll content (Gitelson *et al.* 1999; Buschmann 2007). Equation 8 above provides a functional explanation for such an effect, which is not the result of leaf absorbance alone, but also of the dilution of excitation among a greater number of chlorophyll molecules with increasing

antenna size, resulting in a lower photochemical quenching and in higher fluorescence rates.

Although both PSII closure and quenching of core antenna complexes are known to have an effect on chlorophyll fluorescence, they are not explicitely included in Eq. 8 above. This is because of the concurrent effects they also have on J_{e^-}, making it possible to enucleate the functional link between fluorescence and PSII electron transport (and therefore photosynthetic rates).

The practical application of the preceding equation, however, is hindered by the number of parameters involved. In particular, PSII density is known to change widely over the season and in response to environmental conditions (Laisk *et al.* 2002; Eichelmann *et al.* 2005). However this will also reflect in changes in photosynthetic potential (Eichelmann *et al.* 2004), leading to a more useful form of the preceding equation.

Under saturating light conditions (i.e. for I →∞), all chlorophyll molecules will be in an excited state (i.e. $Chla^{ON} = Chla$), so that maximum electron transport rate under saturating light conditions ($J_{e^-}^{\max}$) can be computed from Eq. 6 as:

$$J_{e^-}^{\max} = \lim_{I \to \infty} J_{e^-} = N_{PSII} \cdot \frac{\gamma \cdot k_p \cdot Q}{k_p + \gamma \cdot Q} \tag{9}$$

By substituting this expression into Eq. 8, fluorescence rates can be expressed as a function of instantaneous and potential electron transport rates as:

$$f = \frac{k_f}{k_p} \cdot Chla \cdot \frac{1}{\frac{1}{J_{e^-}} - \frac{1}{J_{e^-}^{\max}} \cdot \frac{k_p}{k_p + \gamma \cdot Q}} \tag{10}$$

3.2.5 Derivation of rate constants and model parameterisation

The numerical value of the rate constants (k_p, k_f) presented in the model can be derived from the literature or from simple relationships.

In a dark-acclimated leaf, xanthophyll de-epoxidation can be assumed to be fully dissipated. The quinone-equivalent pool will also be fully oxidized (Q^{OFF} / Q = 1; all reaction centres are open), so that the fluorescence yield (ΦF_o) of such a dark-adapted leaf (as typically measured by a modulated fluorometer under a dim measuring light) will be (Krause et al. 1991; Lavergne et al. 1995):

$$\Phi F_o = \frac{k_f}{k_d + k_f + \frac{k_p}{Chla}} \tag{11}$$

where k_d (s^{-1}) is the rate constant of constitutive heat dissipation.

After exposing the leaf to a brief pulse of saturating light, the quinone-equivalent pool can be assumed to be fully reduced (Q^{OFF} / Q = 0; all reaction centres are closed), and the maximum dark-acclimated fluorescence yield (ΦF_m) can be expressed as (Krause *et al.* 1991):

$$\Phi F_m = \frac{k_f}{k_d + k_f} \tag{12a}$$

$$k_d = k_f \left(\frac{1}{\Phi F_m} - 1 \right) \tag{12b}$$

Since the maximum fluorescence yield for molecules associated with photosystem II *in vivo* is typically 10% (Barber *et al.* 1989), and the rate constant for fluorescence is 6.7×10^7 s^{-1} (Rabinowich *et al*. 1969), the rate constant for constitutive heat dissipation (k_d) can be estimated from Eq. 12. Finally, the yield

of photochemistry of a dark-acclimated leaf (Φ*PSII*) can be expressed as (Krause *et al.* 1991):

$$\Phi PSII = \frac{k_p / Chla}{k_d + k_f + k_p / Chla} \tag{13a}$$

$$k_p = \frac{\left(k_d + k_f\right) \cdot Chla}{\frac{1}{\Phi PSII} - 1} \tag{13b}$$

In healthy leaves, Φ*PSII* and *Chl* have typical values of 0.88 (Pfundel 1998) and 148 (Amerongen *et al.* 2003), respectively, the value of the intrinsic rate constant for photochemistry (k_p) can be estimated from Eq. 13. A value of 22 has been suggested for *Q*, the number of quinone-equivalents per PSII (Porcar-Castell *et al.* 2006). The rate constant for the re-oxidation of the quinone-equivalent pool can be attributed a value of 175 s^{-1}, equivalent to the slowest step in the quinone redox cycle (Zhu *et al.* 2005). Values for all rate constants are presented in Table 1.

Using these numerical values, it can be seen that $k_p >> \gamma \cdot Q$, so that Eqn. 10 can be safely approximated as:

$$f = \frac{k_f}{k_p} \cdot Chla \cdot \frac{1}{\frac{1}{J_{e-}} - \frac{1}{J_{e-}^{\max}}} \tag{10a}$$

Finally, it can be seen from Eq. 11 and 13 above that it holds:

$$\frac{k_p}{k_f \cdot Chla} = \frac{\Phi PSII}{\Phi F_o} \tag{14}$$

so demonstrating the rationale for normalizing fluorescence measurements (Flexas *et al.* 2002; Evain *et al.* 2004).

3.2.6 Comparison with modulated fluorescence data

Chlorophyll fluorescence and photochemical quantum yields are routinely measured under field and laboratory conditions by the so-called pulse-saturated technique, which combines records under ambient conditions (F_t) with the measurement of fluorescence in response to a short (1s) flash of saturating light (F_m'). This makes it possible to eliminate one of the three terms of the energy balance, estimating the remaining two from each other and deduce the electron transport rate and the photochemical quantum yield as (Genty et al. 1989):

$$\frac{\Delta F}{F_m'} = \frac{F_m' - F_t}{F_m'} \tag{15}$$

Photosynthetic electron transport rates (J_{e^-}) can then be estimated from fluorescence measurements as:

$$J_{e^-} = \frac{\Delta F}{F_m'} \cdot PAR \cdot \alpha \cdot 0.5 \tag{16}$$

where *PAR* is photochemically active radiation at the leaf surface, α is leaf absorbance (assumed not to change over the course of the experiment) and the factor 0.5 accounts for light partitioning between photosystems.

Photosystem II fluorescence yield (Φ*F*, the fraction of PSII absorbed light dissipated as fluorescence) can also be estimated from the same set of measurements. In the widely applied modulated fluorometer, fluorescence under ambient conditions (as well as under saturating conditions) is determined from

the periodic changes in re-emitted near-infrared light induced by a dim modulated (0.6 kHz) light, the so-called measurement light (I_{meas}).

Since the measurement light is of constant intensity and so weak as not to interfere with PSII status, the fluorescence signal is proportional to fluorescence yield under background light conditions:

$$\Phi F = \frac{F_t}{I_{meas} \cdot \alpha \cdot 0.5} \tag{17}$$

and fluorescence radiance (f) therefore estimated as:

$$f = \frac{F_t}{I_{meas} \cdot \alpha \cdot 0.5} \cdot \alpha \cdot 0.5 \cdot PAR = F_t \cdot \frac{PAR}{I_{meas}} \tag{18}$$

It is therefore possible to obtain concurrent estimates of fluorescence radiance (f) and electron transport rates ($J_{e\text{-}}$), as assessed by the saturating pulse technique, and evaluate their mutual relationship.

3.2.7 Correction for PSI contributions

It is important to remember, however, that measurements from commercial modulated fluorometers also include a substantial contribution from photosystem I (PSI; Pfundel 1998; Franck *et al.* 2002), which should be subtracted in order to obtain results fully comparable with model predictions of PSII yields. In contrast with PSII, the contribution from PSI (F^I) appears to be independent from the state of its reaction centre and non-photochemical quenching (Butler 1978). The

modulated fluorescence signal from a dark-acclimated leaf before (F_o) and after (F_m) a flash of saturating light can be therefore expressed as:

$$F_o = F^I + F_o^{II} \tag{19}$$

$$F_m = F^I + F_m^{II} \tag{20}$$

where F_o^{II} and F_m^{II} are the contributions from PSII alone. Variable fluorescence from such a dark-acclimated leaf (F_v) can be therefore expressed as:

$$F_v = F_m - F_o = F_m^{II} - F_o^{II} \tag{21}$$

Maximum quantum yield is commonly estimated as (Genty *et al.* 1989):

$$\frac{F_v}{F_m} = \frac{F_m^{II} - F_o^{II}}{F^I + F_m^{II}} \tag{22}$$

and is commonly found to have a value of 0.84 (Schreiber *et al.* 1987). Because of the contribution from PSI, this is lower than the 0.88 value estimated for the maximum photochemical yield of PSII alone (Pfundel 1998):

$$\Phi PSII = \frac{F_m^{II} - F_o^{II}}{F_m^{II}} \tag{23}$$

From the two preceding equations, PSI fluorescence can be estimated from measurements on a dark-acclimated leaf as:

$$F^{I} = F_o \cdot \left(\frac{1}{F_v/F_m} - \frac{1}{\Phi_{PSII}} \right) \cdot \frac{F_v/F_m}{1 - F_v/F_m} \quad (24)$$

and used to correct the estimates of PSII fluorescence radiance and electron transport rates derived from modulated fluorescence measurements.

3.3 Material and methods

3.3.1 Plant material

Five strawberry tree (*Arbutus unedo* L.) and five hybrid poplar (*Populus x euroamericana*) plants were grown in pots of 11.3 l capacity containing a mixture of sand and peat (1:1, by volume). The pots were maintained during the experiment period in an open area at the Faculty of Agriculture of the University of Bologna with adequate watering supply.

3.3.2 Experimental procedures

The experiment was conducted in the laboratory of ecophysiology of the DCA in the Faculty of Agriculture, University of Bologna (Italy). Before each set of measurements, the plant was exposed to high light conditions for one hour in order to acclimate the leaves and activate the photosynthesis process.

Measurements under controlled conditions were repeated with the same experimental setup on seven fully expanded mature leaves from separate plants for each species and protocol.

The leaf was enclosed in the broadleaf cuvette of a LiCor Li-6400 gas-exchange analyzer (LI-Cor Inc., Lincoln, NE, USA), which also provided gas exchange measurements, and exposed to the light of a dichroic halogen lamp. The lamp was connected to an energy stabilizer in order to provide the light intensity needed. Simultaneously chlorophyll fluorescence parameters were measured by pointing the fiberoptics probe of a PAM-2000 modulated fluorometer (Heinz Walz

Gmbh, Effeltrich, Germany) at the surface of the leaf through a sealed opening in the leaf cuvette.

Environmental conditions applied on leaves in the LiCor cuvette were varied one at a time, following two different protocols. In the first protocol, light intensity were changed in steps (1000, 800, 600, 400, 200, 100, 20 μmol m^{-2} s^{-1}) from high to low level, whilst keeping ambient CO_2 inside the cuvette constant (350 ppm). The same steps were then repeated at high (800 ppm) and low (100 ppm) air CO_2 concentration inside the cuvette. In the second protocol, the air CO_2 concentration inside the LiCor Li-6400 cuvette were changed in steps (350, 200, 50, 350, 550, 800, 1200, 1500 ppm), under moderately low (400 μmol m^{-2} s^{-1}) and high (1000 μmol m^{-2} s^{-1}) light conditions.The leaf was kept for at least 15 minutes at each level in order to ensure full acclimation. At the end of each step, three successive measurements were made by the LiCor Li-6400 at 2-m intervals, simultaneously with fluorescence measurements by the PAM 2000. Temperature inside the cuvette of the LiCor Li-6400 was maintained at 25 ± 1°C throughout the experiment. A more detailed description of experimental setup can be found in Chapter 2.

3.3.3 Model validation

In order to test its predictive power, the model described above has been fitted on fluorescence measurements by the SAS statistical package (SAS 9.0, SAS Institute Inc., Cary, NC, USA), using a non-linear regression procedure (PROC NLIN) and a derivative-free algorithm (Ralston *et al.* 1978). Values for most parameters were derived from the literature (see Table 1). Modulated fluorescence from the dark-acclimated leaf, required for the correction of the fluorescence contribution from PSI, was directly measured. A single parameter ($J_{e-}^{\max}$) was estimated for each species; additionally, a single value of the I_{meas} parameter (fluorometer modulated measurement light) was fitted on the entire dataset.

Table 1. Values of the rate constants of fluorescence (k_f), constitutive heat dissipation (k_d), pH-dependent non-photochemical heat dissipation (k_n), photochemistry (k_p), plastoquinone pool re-oxidation (γ), of the average number of Chl a molecules per PSII reaction centre (*Chla*), of leaf absorbance (α) and of the fraction of photons partitioned to PSII (β). Additional parameters were needed in the correction for the contribution from photosystem I: the maximum apparent quantum yield with ($\Phi PSII$) or without (F_v/F_m) correction for PSI contribution to fluorescence, and steady-state fluorescence from a dark-acclimated leaf (F_o).

Rate constant or parameter	Value	Source
k_f (s^{-1})	6.7×10^7	Rabinowich and Govindjee (1969)
k_d (s^{-1})	6.03×10^8	Eq. 12
k_p (s^{-1})	7.3×10^{11}	Eq. 13
γ (s^{-1})	175	Zhu *et al.* (2005)
Chla (-)	148	Amerongen *et al.* (2003)
Q (-)	22	Porcar-Castell *et al.* (2006)
α (-)	0.9	Raddi *et al.*, unpublished
β (-)	0.5	Farquhar *et al.* (1980)
$\Phi PSII$	0.88	Pfundel (1998)
F_v/F_m	0.84	Schreiber *et al.* (1987)
F_o		measured

3.4 Results and discussion

Experimental results from the two species, in terms of photochemical and fluorescence yields and their response to the environment, have been presented in Chapter 2.

Estimated PSII fluorescence radiance and electron transport rates varied widely in response to variable PAR and air CO_2 in both species (Figs. 2 and 3). Maximum electron transport rates under saturating light conditions were about 195 μmol m^{-2} s^{-1} in *A. unedo* (Fig. 2B); in *P. x euroamericana*, electron transport rates ar a PAR of 1000 □mol m^{-2} s^{-1} were as high as 247 μmol m^{-2} s^{-1}, with no clear sign of saturation under conditions of high air CO_2 (Fig. 3B).

Estimated fluorescence radiance responded almost linearly to irradiance in both species, with comparable maximum values of about 4 □mol m^{-2} s^{-1}; the response to air CO2 was weaker in strawberry tree (Fig. 2A) than in poplar (Fig. 3A). As air CO_2 declined to values close to the compensation point for photosynthesis, both electron transport rates and PSII fluorescence radiances did not approach zero in these C_3 species, as a result of the sustained demand for ATP by oxygenation processes in photorespiration (Farquhar *et al.* 1980). As a result, the observed variability in both *f* and J_{e^-} was much more strongly controlled by absorbed light than by net photosynthesis per se; changes in air CO2 (which could mimic the functional effects of stomatal closure under stress conditions) were the key driver of both fluxes only under high light conditions. Although this should be kept in mind in the analysis of leaf-level data, it should not create a problem in a remote sensing perspective, since satellite acquisitions are commonly made at around midday and under clear conditions (Joiner *et al.* 2011), when radiation levels are high during summer months when stress conditions are commonly observed.

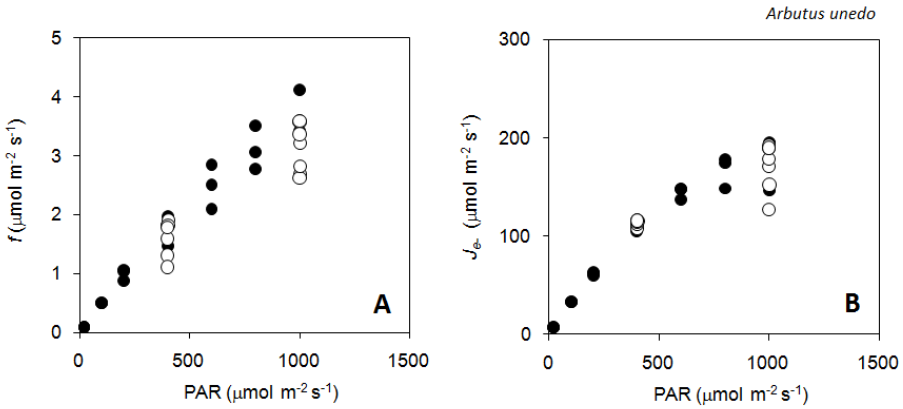

Fig. 2 Response of estimated (**A**) fluorescence radiance (*f*) and (**B**) photosynthetic electron transport rate (J_{e^-}) to incoming irradiance (PAR) in *A. unedo* leaves submitted to the first (variable PAR at three levels of CO_2; black dots) and to the second protocol (variable CO_2 at two levels of PAR; white circles)

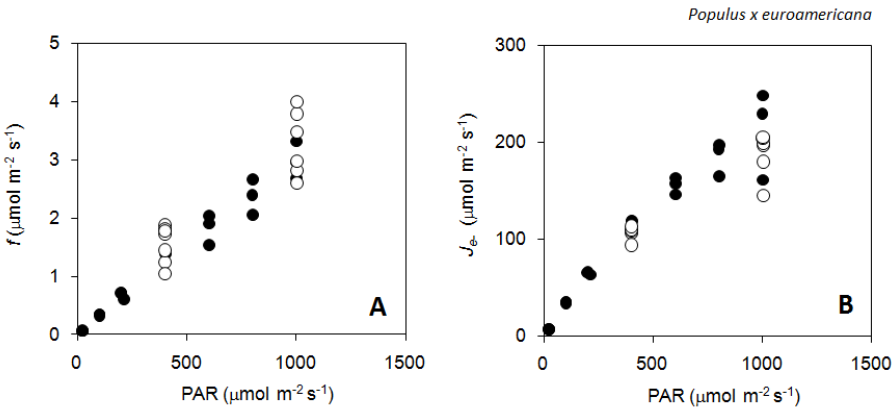

Fig. 3 Response of estimated (**A**) fluorescence radiance (*f*) and (**B**) photosynthetic electron transport rate (J_{e^-}) to incoming irradiance (PAR) in *P. x euroamericana* leaves submitted to the first (variable PAR at three levels of CO_2; black dots) and to the second protocol (variable CO_2 at two levels of PAR; white circles)

Considering the mutual relationship between fluorescence radiance and electron transport rate, a curvilinear relationship is observed both in *Arbutus* (Fig. 4A) and in *Populus* (Fig. 5A). For each species, a unique relationship is observed between the two variables, irrespective of whether the variation in electron transport rates is induced by changes in either incoming irradiance (black dots) or in atmospheric CO_2 concentrations (white circles). The uniqueness of the relationship, predicted by the fluorescence model, is most pronounced in strawberry tree leaves, whilst some differences between results from the two

protocols are apparent in poplar. Despite this partial discrepancy, the model appears to explain well the overall relationship between the two variables (Figs 4B and 5B), opening the way to the prediction of photosynthetic electron transport from measurements of steady-state fluorescence alone. In both species, the model explains 97% of the overall variability in fluorescence radiance; model predictions are on average 13% higher than direct estimates in the case of poplar, but 6% lower in strawberry trees. This contrasts with the rather poor performance of the steady-state fluorescence model presented by van der Tol et al. (2008), when tested against the laser-induced fluorescence measurements of Rosema *et al.* (1998) on *P. nigra* leaves subjected to diurnal changes of irradiance and to increasing water stress conditions: the van der Tol model only explained 84% of the overall variability in fluorescence radiance. Even poorer results were obtained by the original model of Rosema *et al.* (1998), which contained several elements of empiricism and was not fully consistent with our general understanding of the relationship between fluorescence and photochemistry (Genty *et al.* 1989; Kramer *et al.* 2004).

A comparison of results and model predictions from the two species is also rather informative (Fig. 6): there appears to be a general consistence between the two species in the relationship, but not such that it can be used as an empirical tool for the prediction of photochemical rates. This is explained by the model in terms of a single parameter ($J_{e-}^{\max}$), which according to Eq. 9 should be related to the number of PSII units per unit leaf area (N_{PSII}), as well as to a number of other parameters (k_p, Q, γ) which have been assumed as constant in the current analysis, but which could vary between species and in response to stress conditions. It is worth noting that a higher value of the parameter has been estimated by the model in the case of poplar, a species typically characterized by high leaf N contents, photosynthetic potentials and (presumably) a high density of PSII units.

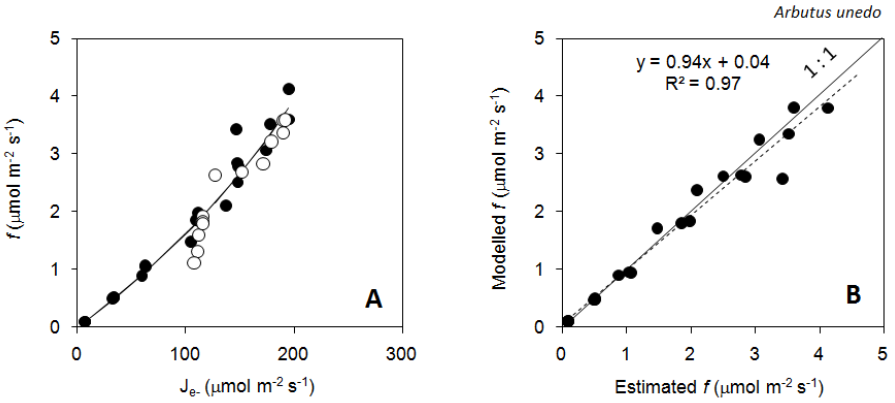

Fig. 4 Analysis of fluorescence model goodness-of-fit in *A. unedo* leaves. (**A**) Predicted (continuous line) and estimated (protocol 1, black dots; protocol 2, white circles) relationship between fluorescence radiance (*f*) and photosynthetic electron transport rate ($J_{e\text{-}}$). (**B**) Comparison between modelled and estimated values of fluorescence radiance (f); linear regression (dotted line) and 1:1 line (continuous line) are also presented.

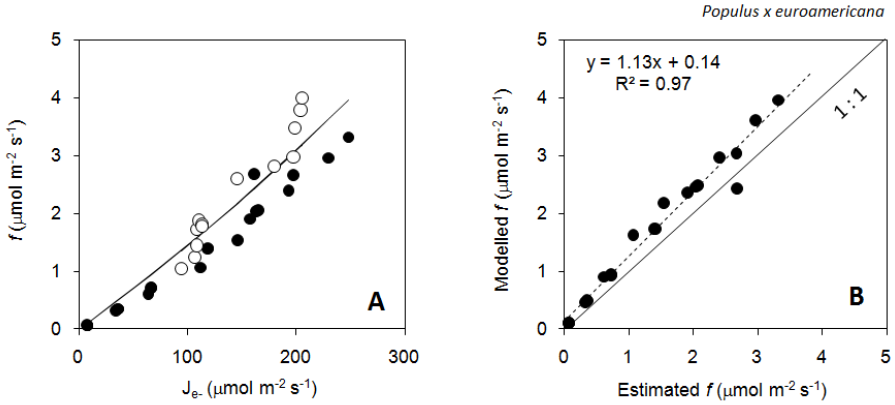

Fig. 5 Analysis of fluorescence model goodness-of-fit in *P. x euroamericana* leaves. (**A**) Predicted (continuous line) and estimated (protocol 1, black dots; protocol 2, white circles) relationship between fluorescence radiance (*f*) and photosynthetic electron transport rate ($J_{e\text{-}}$). (**B**) Comparison between modelled and estimated values of fluorescence radiance (f); linear regression (dotted line) and 1:1 line (continuous line) are also presented.

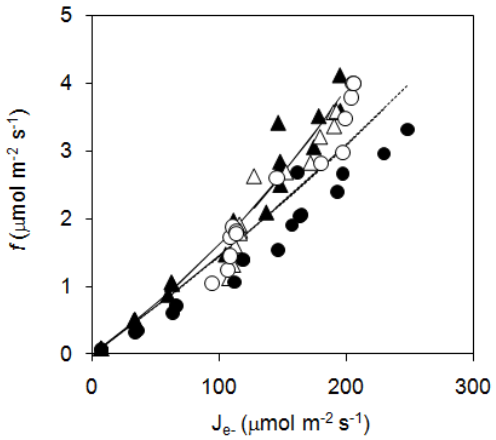

Fig. 6 Inter-specific comparison of the relationship between fluorescence radiance (*f*) and photosynthetic electron transport rate (J_{e^-}). Modelled (continuous line, *A. unedo*; dotted line, *P. x euroamericana*) and estimated values (triangles, A. unedo; circles, P. x euroamericana) are presented for protocol 1 (black) and protocol 2 (white).

3.5 Conclusions

The measurement of ambient fluorescence has been proposed as a suitable tool for the remote sensing of vegetation primary production from airborne and satellite platforms.

The assessment of ambient fluorescence from space is generally based on the retrieval of solar-induced fluorescence radiance through the in-filling of the so-called Fraunhofer lines (in reality, the O_2-A and O_2-B atmospheric absorption lines). Two different methods have been proposed for this purpose in the literature (Alonso *et al.* 2008; Frankenberg *et al.* 2011).

At the leaf level, solar-induced fluorescence radiance can also be measured and discriminated from near-infrared reflectance through special experimental set-ups, by screening all wavelengths above 680 nm from incoming radiation, so as to measure by a spectro-radiometer only outgoing fluorescence in this spectral region (Meroni *et al.* 2009).

An alternative, simple approach has been presented here, based on a widely available modulated fluorometer and on the interpretation of the F_t signal in terms of fluorescence yield. Although the method does not provide a direct measurement of fluorescence radiance, which has to be estimated taking radiation absorption into account, it has several advantages over other techniques, by (i) making it possible to concurrently estimate electron transport rates from the same portion of the leaf and (ii) making it easy to expose the leaf to controlled conditions using commercial instrumentation. This opens the way for a thorough, one-at-a-time test of process based models of leaf energy dissipation and fluorescence, such as the one presented in this chapter. For the first time, and in contrast with previous studies (Rosema *et al.* 1998; van der Tol *et al.* 2008), a quantitative test of the model has been made possible by the proposed experimental setup.

The proposed model differs substantially from previous approaches. In contrast with most models of chlorophyll fluorescence and leaf photochemistry (Genty *et al.* 1989; Hendrickson *et al.* 2004; Kramer *et al.* 2004), the model relies for the prediction of electron transport rates only on knowledge of ambient fluorescence, making it suitable for the interpretation of solar-induced fluorescence from space. In comparison with previous attempts to explain the Ft signal (Rosema *et al.* 1998; van der Tol *et al.* 2008), the new model does not rely on empirical un-warranted assumptions, such as the parallel limitation of fluorescence and photochemistry by non-radiative energy dissipation, but is based on purely biophysical principles.

The test has highlighted the general validity of the model, which appears to conveniently represent the relationship between fluorescence radiance and electron transport rates, when the latter is varied in response to either light or atmospheric CO_2. This paves the way for the retrieval of electron transport rates from remotely-sensed information. A precise knowledge of J_{e^-} is the starting point for an assessment of photosynthetic rates at leaf and canopy level (despite all the caveats highlighted in the present study, centered on the effects of photorespiration). As a result, the model represents an important step forward for the estimation of global vegetation productivity from remote sensing measurements of solar-induced fluorescence.

Appendix 1. Developing an expression for chlorophyll fluorescence

Recalling Eq. 4 and 5, the rates of PSII excitation and de-excitation can be expressed alternatively as:

$$p = J_{e-} = k_p \cdot \frac{Q^{OFF}}{Q^{OFF} + Q^{ON}} \cdot \frac{Chla^{ON}}{Chla} \cdot N_{PSII} \tag{4}$$

$$J_{e-} = \gamma \cdot Q^{ON} \cdot N_{PSII} \tag{5}$$

From Eq. 5, the number of reduced quinone-equivalent molecules per PSII can be expressed as a function of electron transport rate J_{e-}:

$$Q^{ON} = \frac{J_{e-}}{\gamma \cdot N_{PSII}} \tag{A1.1}$$

By combining the preceding equation with Eq. 4, electron transport rate can be expressed as a function of the number of excited chlorophyll *a* molecules per PSII:

$$J_{e-} = k_p \cdot \left(1 - \frac{Q^{ON}}{Q}\right) \cdot \frac{Chla^{ON}}{Chla} \cdot N_{PSII} \tag{A1.2a}$$

$$J_{e-} = k_p \cdot \left(1 - \frac{J_{e-}}{\gamma \cdot N_{PSII} \cdot Q}\right) \cdot \frac{Chla^{ON}}{Chla} \cdot N_{PSII} \tag{A1.2b}$$

$$J_{e-} = N_{PSII} \cdot \frac{k_p \cdot \frac{Chla^{ON}}{Chla} \cdot \gamma \cdot Q}{\gamma \cdot Q + k_p \cdot \frac{Chla^{ON}}{Chla}} \tag{A1.2c}$$

Alternatively, the number of excited chlorophyll *a* molecules per PSII can be expressed as a function of electron transport rate:

$$Chla^{ON} = Chla \cdot \frac{J_{e-}}{k_p \cdot N_{PSII} \cdot \left(1 - \frac{J_{e-}}{\gamma \cdot N_{PSII} \cdot Q}\right)} \quad \text{(A1.3a)}$$

$$Chla^{ON} = \frac{Chla}{k_p} \cdot \frac{\gamma \cdot Q \cdot J_{e-}}{\gamma \cdot N_{PSII} \cdot Q - J_{e-}} \quad \text{(A1.3b)}$$

By combining the preceding equation with Eq. 3, which represents the dependence of fluorescence rates upon the number of excited chlorophyll *a* molecules, fluorescence *f* can be also expressed as a function of electron transport rates:

$$f = \frac{k_f}{k_p} \cdot Chla \cdot \frac{N_{PSII} \cdot \gamma \cdot Q \cdot J_{e-}}{N_{PSII} \cdot \gamma \cdot Q - J_{e-}} \quad \text{(A1.4)}$$

As already noted, however, the practical application of the preceding equation is hindered by the number of parameters involved. In particular, PSII density is known to change widely over the season (Laisk *et al.* 2002; Eichelmann *et al.* 2005). This will also reflect in changes in photosynthetic potential, however, leading to a more useful form of the preceding equation.

Under saturating light conditions, all chlorophyll molecules will be in an excited state ($Chla^{ON} = Chla$), so that maximum electron transport rate under saturating light conditions can be computed from Eq. A1.2 as:

$$J_{e-}^{\max} = N_{PSII} \cdot \frac{\gamma \cdot k_p \cdot Q}{k_p + \gamma \cdot Q} \quad \text{(A1.5)}$$

Alternatively, the electron transport rate with all reaction centres closed $\left(= N_{PSII} \cdot \gamma \cdot Q\right)$ can be estimated as:

$$N_{PSII} \cdot \gamma \cdot Q = J_{e-}^{\max} \cdot \left(1 + \frac{\gamma \cdot Q}{k_p}\right) \tag{A1.6}$$

By substituting this expression into Eq. A1.4, fluorescence rates can be expressed as a function of instantaneous and potential electron transport rate as:

$$f = \frac{k_f}{k_p} \cdot Chla \cdot \frac{J_{e-}^{\max} \cdot \left(1 + \frac{\gamma \cdot Q}{k_p}\right) \cdot J_{e-}}{J_{e-}^{\max} \cdot \left(1 + \frac{\gamma \cdot Q}{k_p}\right) - J_{e-}} \tag{A1.7a}$$

$$f = \frac{k_f}{k_p} \cdot Chla \cdot \frac{1}{\frac{1}{J_{e-}} - \frac{1}{J_{e-}^{\max}} \cdot \frac{k_p}{k_p + \gamma \cdot Q}} \tag{A1.7b}$$

Reference List

Alonso L, Gomez-Chova L, Vila-Frances J, Amoros-Lopez J, Guanter L, Calpe J, Moreno J(2008) Improved Fraunhofer line discrimination method for vegetation fluorescence quantification. *IEEE Transactions in Geoscience and Remote Sensing* **5** 620-624.

Amerongen H, Dekker JP (2003) Light-harvesting in photosystem II. In:Light-Harvesting Antennas in Photosynthesis (eds Green BR, Parson WW), pp. 219-251. Kluwer Academic, Dordrecht.

Baker NR(2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annual Review of Plant Biology* **59** 89-113.

Barber J, Malkin S, Telfer A(1989) The origin of chlorophyll fluorescence *in vivo* and its quenching by the photosystem II reaction centre. *Philosophical Transactions of the Royal Society of London, Series B* **323** 227-239.

Bernhardt K, Trissl HW(1999) Theories for kinetics and yields of fluorescence and photochemistry: how, if at all, can different models of antenna organisation be distinguished experimentally? *Biochimica et Biophysica Acta* **1409** 125-142.

Buschmann C(2007) Variability and application of the chlorophyll fluorescence emission ratio red/far-red of leaves. *Photosynthesis Research* **92** 261-271.

Butler WL(1978) Energy distribution in the photochemical apparatus of photosynthesis. *Annual Review of Plant Physiology* **29** 345-378.

Dau H(1994) Molecular mechanisms and quantitative models of variable photosystem II fluorescence. *Photochemistry & Photobiology* **60** 1-23.

Eichelmann H, Oja V, Rasulov B, Padu E, Bichele I, Pettai H, Mand P, Kull O, Laisk A(2005) Adjustment of leaf photosynthesis to shade in a natural canopy: reallocation of nitrogen. *Plant Cell and Environment* **28** 389-401.

Eichelmann H, Oja V, Rasulov B, Padu E, Bichele I, Pettai H, Niinemets U, Laisk A(2004) Development of leaf photosynthetic parameters in *Betula pendula* Roth leaves. Correlations with Photosystem I density. *Plant Biology* **6** 307-318.

Evain S, Flexas J, Moya I(2004) A new instrument for passive remote sensing. 2. Measurement of leaf and canopy reflectance changes at 531 nm and their relationship with photosynthesis and chlorophyll fluorescence. *Remote Sensing of Environment* **91** 175-185.

Farquhar GD, von Caemmerer S, Berry JA(1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149** 78-90.

Flexas J, Escalona JM, Evain S, Gulias J, Moya I, Osmond CB, Medrano H(2002) Steady-state chlorophyll fluorescence (F_s) measurements as a tool to follow variations of net CO_2 assimilation

and stomatal conductance during water-stress in C-3 plants. *Physiologia Plantarum* **114** 231-240.

Franck F, Juneau P, Popovic R(2002) Resolution of the Photosystem I and Photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature. *Biochimica et Biophysica Acta* **1556** 239-246.

Frankenberg C, Butz A, Toon GC(2011) Disentangling chlorophyll fluorescence from atmospheric scattering effects in O2 A-band spectra of reflected sun-light. *Geophysical Research Letters* **38** L03801.

Genty B, Briantais J-M, Baker NR(1989) The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* **990** 87-92.

Gilmore AM, Shinkarev VP, Hazlett TL, Govindjee(1998) Quantitative analysis of the effects of intra-thylakoid pH and xanthophyll cycle pigments on Chlorophyll a fluorescence lifetime distributions and intensity in thylakoids. *Biochemistry* **37** 13582-13593.

Gitelson A, Buschmann C, Lichtenthaler HK(1999) The chlorophyll fluorescence ratio F735/F700 as an accurate measure of the chlorophyll content in plants. *Remote Sensing of Environment* **69** 296-302.

Grace J, Nichol C, Disney M, Lewis P, Quaife T, Bowyer P(2007) Can we measure terrestrial photosynthesis from space directly, using spectral reflectance and fluorescence? *Global Change Biology* **13** 1484-1497.

Guanter L, Alonso L, Gomez-Chova L, Amoros-Lopez J, Vila J, Moreno J(2007) Estimation of solar-induced vegetation fluorescence from space measurements. *Geophysical Research Letters* **34** L08401.

Hendrickson L, Furbank RT, Chow WS(2004) A simple alternative approach to assessing the fate of absorbed light energy using chlorophyll fluorescence. *Photosynthesis Research* **82** 73-81.

Holzwarth AR, Wendler J, Haehnel W(1985) Time resolved picosecond fluorescence spectra of the antenna chlorophylls in *Chlorella vulgaris*. Resolution of photosystem I fluorescence. *Biochimica et Biophysica Acta* **807** 155-167.

Joiner J, Yoshida Y, Vasilkov AP, Yoshida Y, Corp LA, Middleton EM(2011) First observations of global and seasonal terrestrial chlorophyll fluorescence from space. *Biogeosciences* **8** 637-651.

Kitajima M, Butler WL(1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochimica et Biophysica Acta* **376** 105-115.

Kramer DM, Johnson G, Kiirats O, Edwards GE(2004) New fluorescence parameters for the determination of Q(A) redox state and excitation energy fluxes. *Photosynthesis Research* **79** 209-218.

Krause GH, Weis E(1991) Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Physiology and Plant Molecular Biology* **42** 313-349.

Laisk A, Oja V, Rasulov B, Ramma H, Eichelmann H, Kasparova I, Pettai H, Padu E, Vapaavuori E(2002) A computer-operated routine of gas exchange and optical measurements to diagnose photosynthetic apparatus in leaves. *Plant Cell and Environment* **25** 923-943.

Lavergne J, Trissl HW(1995) Theory of fluorescence induction in photosystem II: derivation of analytical expressions in a model including exciton-radical-pair equilibrium and restricted energy transfer between photosynthetic units. *Biophysical Journal* **68** 2474-2492.

Lazar D(1999) Chlorophyll a fuorescence induction. *Biochimica et Biophysica Acta* **1412** 1-28.

Meroni M, Rossini M, Guanter L, Alonso L, Rascher U, Colombo R, Moreno J(2009) Remote sensing of solar-induced chlorophyll fluorescence: Review of methods and applications. *Remote Sensing of Environment* **113** 2037-2051.

Moya I, Cartelat A, Cerovic ZG, Ducruet J-M, Evain S, Flexas J, Goulas Y, Louis J, Meyer S, Moise N, Ounis A(2003) Possible approaches to remote sensing of photosynthetic activity. *IEEE Transactions in Geoscience and Remote Sensing* 588-590.

Pfundel E(1998) Estimating the contribution of Photosystem I to total leaf chlorophyll fluorescence. *Photosynthesis Research* **56** 185-195.

Porcar-Castell A, Bäck J, Juurola E, Hari P(2006) Dynamics of the energy flow through photosystem II under changing light conditions: a model approach. *Functional Plant Biology* **33** 229-239.

Rabinowich E, Govindjee (1969) Photosynthesis. John Wiley and Sons, New York.

Ralston ML, Jennrich RI(1978) Dud, a derivative-free algorithm for nonlinear least squares. *Technometrics* **20** 7-14.

Rosema A, Snel JFH, Zahn H, Buurmeijer WF, Van Hove LWA(1998) The relation between laser-induced chlorophyll fluorescence and photosynthesis. *Remote Sensing of Environment* **65** 143-154.

Schatz GH, Brock H, Holzwarth AR(1987) Picosecond kinetics of fluorescence and absorbance changes in photosystem II particles excited at low photon density. *Proceedings of the National Academy of Sciences of the USA* **84** 8414-8418.

Schreiber U, Bilger W (1987) Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. In:Plant Response to Stress.Functional Analysis in Mediterranean Ecosystems (eds Tenhunen JD, Catarino FM, Lange OL, Oechel WC), pp. 27-53. Springer-Verlag, Berlin.

van der Tol C, Verhoef W, Rosema A(2008) A model for chlorophyll fluorescence and photosynthesis at leaf scale. *Agricultural and Forest Meteorology* **149** 96-105.

van Grondelle R(1985) Excitation energy transfer, trapping and annihilation in photosynthetic systems. *Biochimica et Biophysica Acta* **811** 147-195.

Whitmarsh J, Govindjee (2002) Photosystem II. In:Encyclopedia of Life Sciences MacMillan Publishers, Nature Publishing group.

Zhu XG, Govindjee, Baker NR, deSturler E, Ort DR, Long SP(2005) Chlorophyll a fluorescence induction kinetics in leaves predicted from a model describing each discrete step of excitation energy and electron transfer associated with photosystem II. *Planta* **223** 114-133.

Chapter 4

Response of photochemical processes to N fertilisation in *Populus*, assessed through fluorescence and leaf spectroscopy techniques

4.1. Abstract

Leaf biochemical contents are known to have a substantial effect on photosynthetic rates, largely explaining the response of plants to fertilization. Both the chlorophyll and the nitrogen content of the leaf are known to be affected by nutrient, and in particular by nitrogen supply. In order to complement the analysis of ambient fluorescence as a predictive tool for the remote assessment of photochemical rates and photosynthesis, I therefore explored in my last experiment the effects of N fertilization on the relationship between photochemical and fluorescence yields, already described before.

The suitability of the Photochemical Reflectance Index (PRI) for the prediction of leaf photochemistry was also assessed in the same experiment. Solar-induced fluorescence and PRI (Photochemical Reflectance Index) are considered the two most promising tools for the remote assessment of photosynthetic rates and an objective comparison of their potential and limitations could greatly enhance our ability to apply these novel techniques to the analysis of vegetation.

The analysis demonstrated a consistent relationship between fluorescence and photochemical yields, once data are corrected for the effects of leaf absorbance. The relationship with the PRI signal, on the contrary, was found t be strongly affected by leaf biochemical contents, presumably because of variable xanthophylls contents, and not to be suitable for the remote assessment of leaf photochemistry over longer periods.

4.2. Introduction

Leaf biochemical contents are known to have a substantial effect on photosynthetic rates, largely explaining the response of plants to fertilization. Both the chlorophyll and the nitrogen content of the leaf are known to be affected by nutrient, and in particular by nitrogen supply. In order to complement the analysis of ambient fluorescence as a predictive tool for the remote assessment of photochemical rates and photosynthesis, I therefore explored in my last experiment the effects of N fertilization on the relationship between photochemical and fluorescence yields, already described before.
The suitability of the Photochemical Reflectance Index (PRI; (Gamon et al. 1997) for the prediction of leaf photochemistry was also assessed in the same experiment. Solar-induced fluorescence and PRI (Photochemical Reflectance Index) are considered the two most promising tools for the remote assessment of photosynthetic rates (Grace et al. 2007); (Malenovsky et al. 2009), and an objective comparison of their potential and limitations could greatly enhance our ability to apply these novel techniques to the analysis of vegetation. Ambient fluorescence and PRI have already been compared under both laboratory (Moya et al. 2004); (Evain et al. 2004)and field conditions (Louis et al. 2005), no analysis under carefully controlled conditions has been proposed so far.

4.3. Material and methods

4.3.1. Plant material

Twenty cuttings of poplar (*Populus euramericana*) clone AF2 were planted on March 19, 2010 in pots of 11.3 L capacity containing a mixture of sand and peat (1:1, by vol.). The pots were maintained in Cadriano (The experimental field of the faculty of agriculture/ university of Bologna) with adequate watering supply. Ten pots were fertilized with 100 kg/ha of an agricultural inorganic NPK fertilizer (containing 21% NO3-N, 7% P2O5-P, and 14% K2O-K), whilst the remaining were not fertilized and acted as a control.

When the plants were five months old, the height, stem base diameter, number of leaves, dimensions of twenty leaves per plant were measured on each plant. An allometric relationship between leaf dimensions and leaf area (Barigah et al, 1994) was applied to calculate the area of individual leaves for the twenty leaves chosen, from which the total leaf area per plant was calculated by multiplying the mean leaf area by the number of leaves. Based on a relation developed in a parallel study on one year plants of the same clone, plant biomass was calculated (E. Muzzi, pers. comm.)

4.3.2. Selection of leaves of variable biochemical content

In order to select for physiological measurements leaves spanning the widest possible range of nutrition, leaf chlorophyll and N contents were measured on a large sample (n=96) of leaves on plants from both treatments (see Chapter 5 for further details). The portable chlorophyll meter (SPAD Minolta 502, Minolta LTD., Osaka, Japan) was used to non-destructively measure the chlorophyll (Chl) and N content of the leaves. SPAD has a 0.06-cm^2 measurement area, and calculate an index in 'SPAD units' based on absorbance at 650 and 940 nm. The accuracy of the SPAD is claimed to be 1.0 SPAD units. Five readings and the mean of them were obtained for 50 healthy mature leaves in 8 different plants of each treatment in order to determine the ones with the maximum and the minimum chlorophyll content. Seven leaves of the maximum SPAD readings and seven leaves of the minimum SPAD readings were chosen to be used for further measurements in the experiment. During measurements with SPAD 502, the sensor head was shaded to avoid direct sunlight from reaching the instrument.

Fig. 1 chlorophyll meter (SPAD-502)

4.3.3. Experimental setup and measurements

The experiment was conducted in the laboratory of Ecophysiology of the DCA in the Faculty of Agriculture-University of Bologna (Italy). Measurements were done under controlled conditions on fully expanded mature leaves. The plants were exposed to high light conditions for one hour before the experiment in order to acclimate leaves and activate the photosynthetic process.

Photosynthetic rates were measured by placing individual leaves inside the broadleaf cuvette of a LiCor Li-6400 infrared gas-exchange analyzer (LI-Cor Inc., Lincoln, NE, USA) and exposing them to the light of a dichroic halogen lamp overhead. The lamp was connected to an energy stabilizer in order to provide the light intensity needed. Simultaneously chlorophyll fluorescence parameters were measured by pointing the fiberoptics probe of a PAM-2000 modulated fluorometer (Heinz Walz Gmbh, Effeltrich, Germany) at the surface of the leaf through a sealed opening in the leaf cuvette. The fiberoptics probe of an Ocean Optics USB-2000 spectrometer (Ocean Optics, Dunedin, FL, USA) connected to a controlled light source was also pointing at the same area of the leaf in order to measure leaf reflectance. The USB-2000 spectrometer had samling frequency of 0.4 nm (about 1 nm FWHM resolution) over the range 400-1000 nm, making it suitable for the determination of hyperspectral indices after application of a suitable Savitzky-Golay smoothing filter. In particular, the Photochemical Reflectance Index (PRI; (Gamon et al. 1997) was computed from leaf reflectance at 531 (R_{531}) and 570 nm (R_{570}) as:

$$PRI = \frac{R_{531} - R_{570}}{R_{531} + R_{570}}$$

The setup of the experiment is illustrated in Fig. 2 and 3.

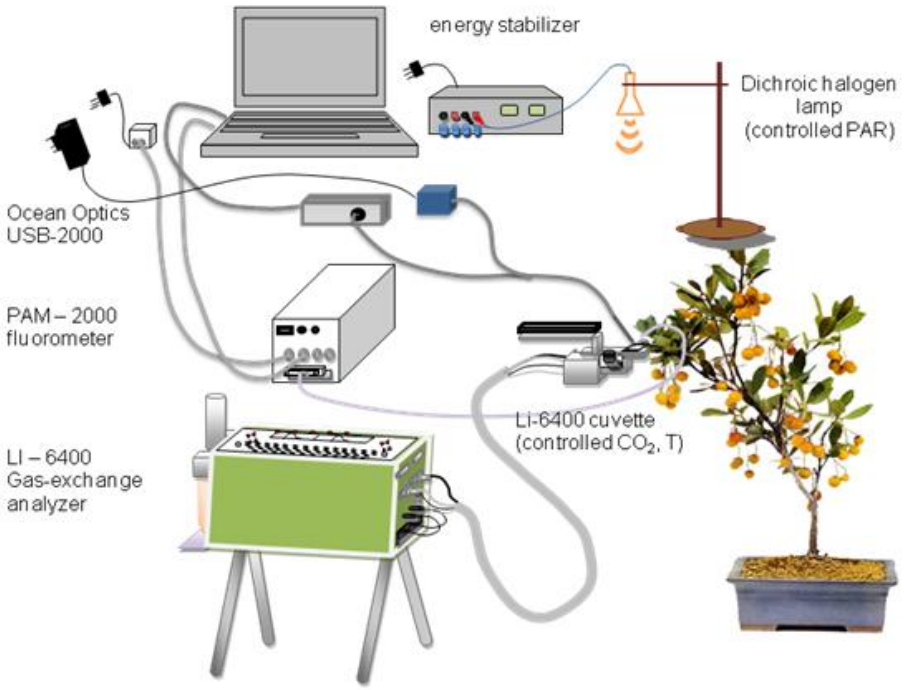

Fig. 2 Set-up scheme of the experiment shows leaf inside the cuvette of the Li-Cor 6400 exposed to the light of a halogen lamp connected to an energy stabilizer; the fiberoptics probe of a PAM-2000 modulated fluorometer is pointing at the surface of the leaf through a sealed opening in the leaf cuvette, and the fiberoptics probe of Ocean Optics USB-2000 spectrometer with a controlled light source is also pointing on the same area of the leaf through the cuvette window.

Fig. 3 The set-up of the experiment: leaf of poplar inside the cuvette (right panel) of the Li-Cor 6400 (left panel, at the bottom) exposed to the light of a halogen lamp connected to an energy stabilizer (left panel, to the left), and the fiberoptics probe of a PAM-2000 modulated fluorometer (left panel, to the right) is pointing at the surface of the leaf through a sealed opening in the leaf cuvette, and the fiberoptics probe of an Ocean Optics USB-2000 spectrometer with a controlled light source is also pointing on the same area of the leaf.

Environmental conditions applied on leaves were varied , changing light levels in steps from 1000 to 20 □mol m^{-2} s^{-1}, whilst keeping air CO_2 concentration constant at 350 ppm (Fig. 4). The same steps were repeated for a moderately high (800 ppm) and moderately low (100 ppm) concentration of air CO_2 in the cuvette. The leaf was kept for at least 15 minutes at each step in order to ensure full acclimation. Fifteen successive measurements were taken at every step by the LiCor Li-6400 at 1-m intervals. The last three measurements were done simultaneously with the PAM 2000 pulse-saturated fluorescence measurements at 2-m intervals. Immediately after the last measurement of fluorescence, the light was turned off and three measurements of leaf reflectance by the Ocean Optics spectrometer were taken at 3-sec intervals. Temperature inside the cuvett of the LiCor Li-6400 was controlled at 25 ± 1°C during all the experiment period.

For each treatment, measurements were repeated with the same experimental setup on seven leaves from separate plants.

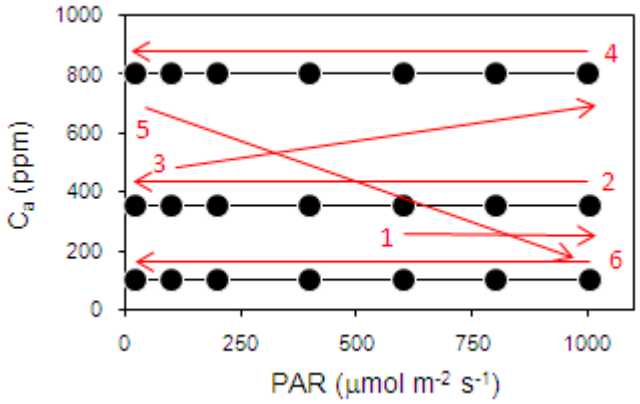

Fig. 4 Schematic representation of the measurement protocol applied on Populus *euroamericana* leaves under controlled conditions: incoming irradiance from a dichroic halogen lamp was modulated through a stabilized power source between 1000 and 20 µmol m^{-2} s^{-1}, whilst maintaining the leaf under three constant CO_2 concentrations (350, 800, 100 ppm) inside the LiCor Li-6400 cuvette.

4.3.4. Estimation of photochemical and fluorescence yield

Chlorophyll fluorescence was measured by the PAM-2000 fluorometer by the so-called pulse-saturated technique, which combines records under ambient

conditions (F_t) with the measurement of fluorescence in response to a short (1s) flash of saturating light (F_m'). This makes it possible to eliminate one of the three terms of the energy balance, estimating the remaining two from each other and deduce the electron transport rate and the photochemical quantum yield as (Genty et al. 1989):

$$\frac{\Delta F}{F_m'} = \frac{F_m' - F_t}{F_m'}$$

Photosystem II fluorescence yield (ΦF, the fraction of PSII absorbed light dissipated as fluorescence) can also be estimated from the same set of measurements. In modulated fluorometers, fluorescence is determined from the periodic changes in re-emitted near-infrared light induced by a dim modulated (0.6 kHz) light, the so-called measurement light (I_{meas}).

Since the measurement light is of constant intensity and so weak as not to interfere with PSII status, the fluorescence signal is proportional to fluorescence yield under background light conditions:

$$\Phi F = \frac{F_t}{I_{meas} \cdot \alpha \cdot 0.5}$$

where α is leaf absorbance of photosynthetically active radiation and the factor 0.5 accounts for light partitioning between photosystems.

It is important to remember, however, that measurements from commercial modulated fluorometers also include a substantial contribution from photosystem I (PSI; (Pfundel 1998); Franck (Franck et al. 2002), which should be subtracted in order to obtain unbiased estimates of photochemical and fluorescence yields. As already discussed (Chapter 3), the contribution to F_t coming from PSI (F^I) can be estimated as:

$$F^I = F_o \cdot \left(\frac{1}{F_v/F_m} - \frac{1}{\Phi_{PSII}} \right) \cdot \frac{F_v/F_m}{1 - F_v/F_m}$$

where Fo is the fluorescence signal from a dark-acclimated leaf, Fv/Fm is the apparent photochemical quantum yield, typically found to have a value of 0.84

(Schreiber et al. 1987) and Φ_{PSII} is the maximum photochemical yield of PSII alone, with a value of 0.88 (Pfundel 1998).

4.3.5. Measurement of leaf absorbance

Leaf absorbance α was measured on a parallel sample of leaves together with directional leaf reflectance by the Ocean Optics spectrometer, so as to be able to estimate it for the leaves measured in the experiment, and correct estimates of fluorescence yield for differences in absorbance (Eq. 2). For this purpose the LICOR LI-1800 (LI-COR, inc., Lincoln, Nebraska, USA) fitted with an 1800-12 external Integrating Sphere (Fig. 5) was used. The instrument measures the reflectance and the transmittance of leaves in a wavelength range between 400 and 1100 nm, and on a spot of diameter 1.14 cm, avoiding the major veins. Leaf PAR absorbance was estimated as the mean of absorbance values in the 400-700 nm region. After the measurement, the leaf was inserted in the cuvette of the Li-6400 gas analyzer, and directional leaf reflectance measured by an Ocean-Optics spectrometer as described above.

Measurements were carried on at the laboratory of ecophysiology in the Faculty of Agriculture of the University of Bologna.

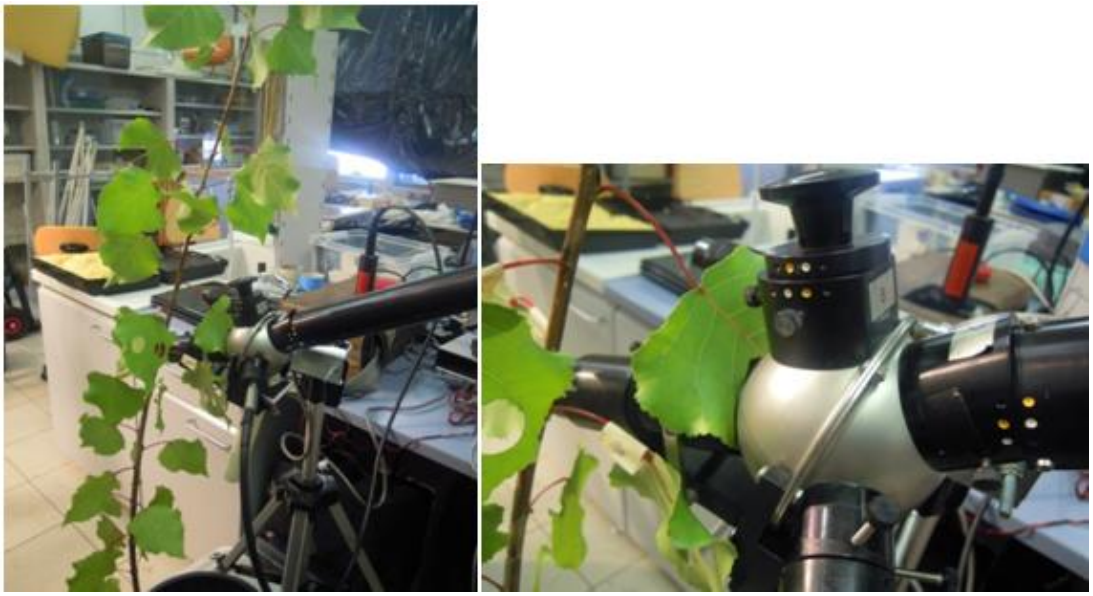

Fig. 5 LICOR LI-1800 provided with 1800-12 external Integrating Sphere

4.4. Results and discussion

The difference between SPAD readings between fertilized and unfertilized populations was rather clear (Fig. 6).

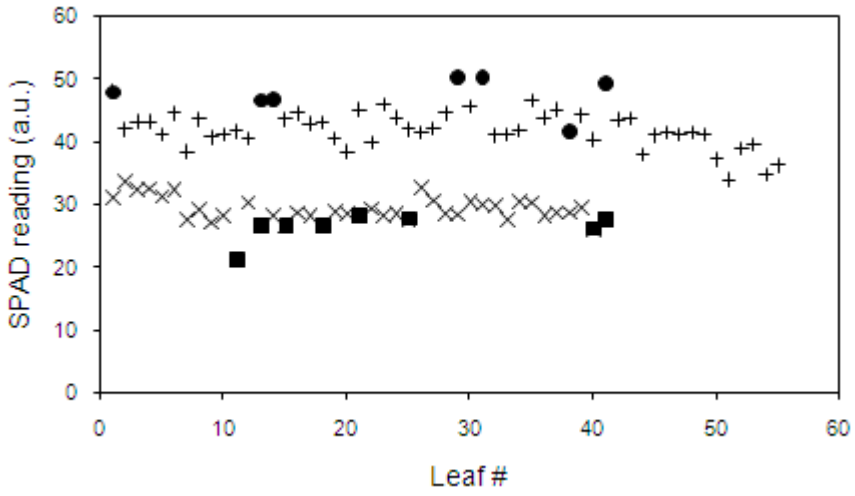

Fig. 6 SPAD readings of leaves of fertilized (upper values) and unfertilized (bottom values) saplings of *Populus euoroamericana*. Leaves used for measurements of fluorescence and reflectance are highlighted: fertilized (●), unfertilized (■)

We found two distinguished populations: fertilized plants were of relatively high values of SPAD readings with an average of about 43, whereas the average was about 29 for unfertilized ones. From each population, a sample of leaves with maximum and minimum biochemical contents was selected (black symbols in Fig. 6).

As a result of the large difference in chlorophyll and carotenoid content captured by the divergent SPAD readings (see Chapter 5), fertilized and unfertilized plants also had widely different light absorbances in the visible range (Fig. 7). Average absorbance of photosynthetically active radiation was 85.8 and 80.7% in selected leaves from fertilized and unfertilized treatments, respectively.

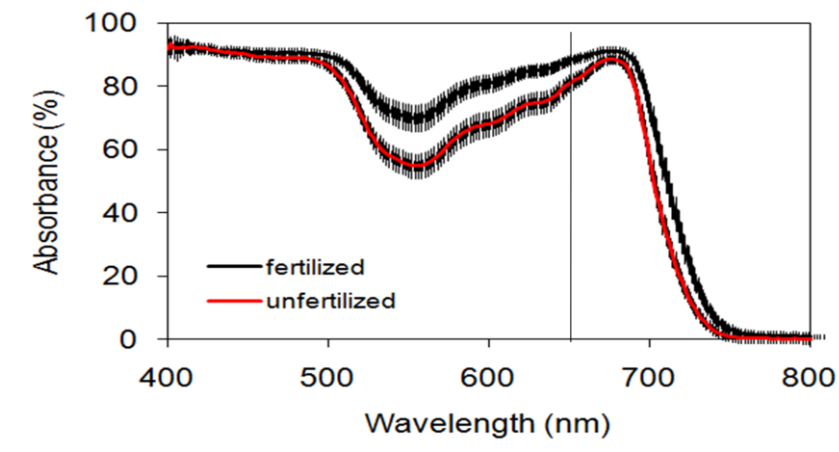

Fig. 7 Leaf absorbance of fertilized (upper curve) and unfertilized (bottom curve) saplings of *P. euroamericana*. Vertical bar shows the wavelength (650 nm) used by SPAD for making instantaneous readings of leaf greenness or relative chlorophyll content.

The relationship between the fluorescence Ft signal and estimated photochemical yield ($\Delta v/F_m'$) shows a clear non-monotonic pattern (Fig. 8). As already observed in previous experiments (see Chapter 2), a negative association between Ft and photochemical yield is observed under light-limited conditions, and a positive association on the contrary under conditions limited by stomatal closure and CO_2 availability. The transition point, as well as the slope of the relationship under CO_2-limited conditions, clearly depend upon air CO2 concentrations, and can be therefore expected to vary in response to drought and stomatal closure (Flexas et al. 2002). Raw data obtained from the PAM-2000 fluorometer show a clear difference in the relationship between fertilized and un-fertilized leaves (Fig. 8). The difference, however, is largely the effect of differences in leaf absorbance, and is not apparent anymore when comparing fluorescence and photochemical yields, computed as described above (Fig. 9); at all three CO2 levels, the segmented relationship between yields is maintained, but results from the two fertilization treatments are undistinguishable from each other.

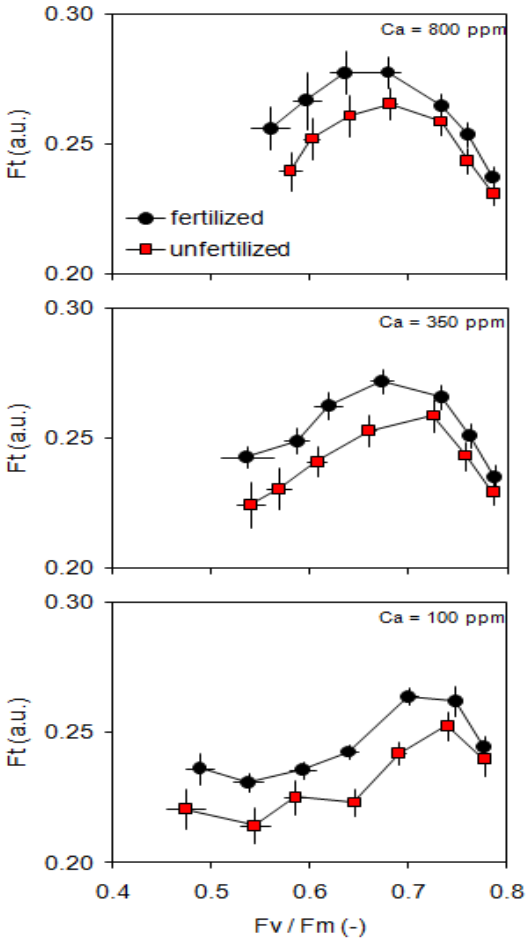

Fig. 8 Relationship between fluorescence yield (Φ_f) and PSII photochemical yield (Φ_{PSII}) (raw data) in response to changes in irradiance (PAR) under three constant CO_2 concentrations in leaves of fertilized (●) and unfertilized (■) plants of *Populus euroamericana*.

No such effects of leaf aborbance can be invoked to explain the large effect of fertilization on leaf PRI (Fig. 10). There is indeed, as expected (Gamon and Surfus 1999); (Garbulsky et al. 2011), a relationship at the different levels of CO_2 and the different levels of fertilization between photochemical yield and PRI; at a CO_2 of 800 and 350 ppm, the relationship was almost linear, but it appeared to level off under low CO_2 conditions under high light (low values of photochemical yield). Lower PRI values were also observed at a CO_2 of 100 ppm for a given level of photochemical yield (Fig. 11).

The relationship between PRI and photochemical yields, however, differed markedly as a result of differences in leaf biochemical contents (Fig. 10), with much lower PRI values in the control than in the fertilized treatment. As a result,

PRI cannot be used as a predictor of photochemical yield over long periods, as already reported in the literature (Garbulsky et al. 2011).

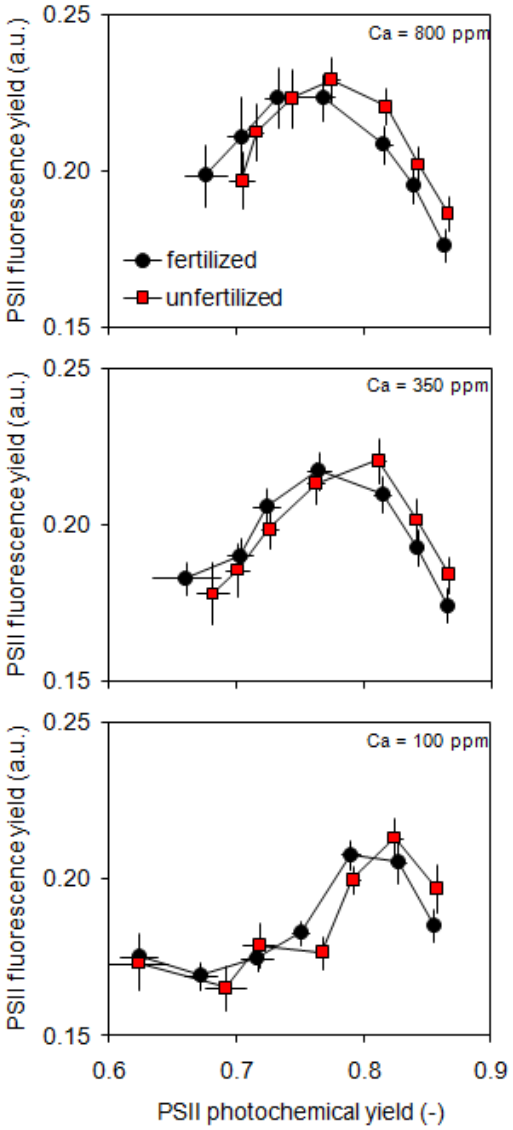

Fig. 9 Relationship between fluorescence yield (Φ_f) and PSII photochemical yield (Φ_{PSII}) (after correction for absorbance) in response to changes in irradiance (PAR) under three constant CO_2 concentrations in leaves of fertilized (●) and unfertilized (■) plants of *Populus euroamericana*.

Over the course of one day, the biochemical content of the leaf will remain constant, and therefore a good relationship can be observed between PRI and photochemical yield (Gamon et al. 1997). Over the long term, however, we will have changes in nitrogen or chlorophyll concentration or other photosynthetic

pigments, and this will have an effect on PRI independent of the effect on photochemical yield. Since it is not possible at present to discriminate between the two components, this appears to limit the applicability of PRI in proximal and remote sensing studies.

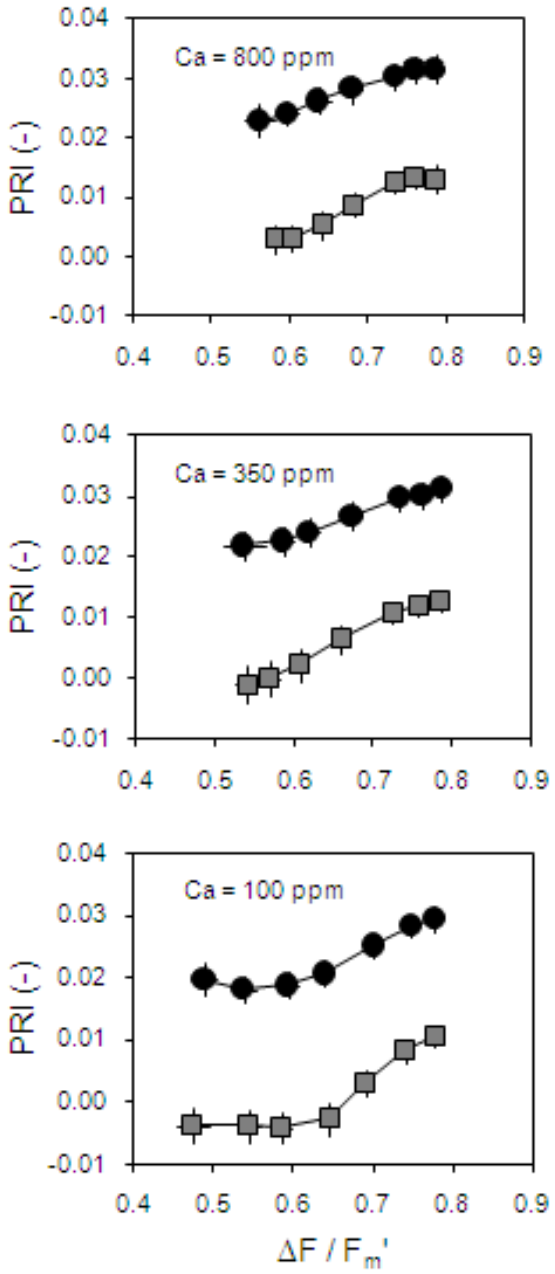

Fig. 10 Relationship between photochemical reflectance index (PRI) and PSII photochemical yield (Φ_{PSII}) in response to changes in irradiance (PAR) under three constant CO_2 concentrations in leaves of fertilized (●) and unfertilized (■) plants of *Populus euroamericana*.

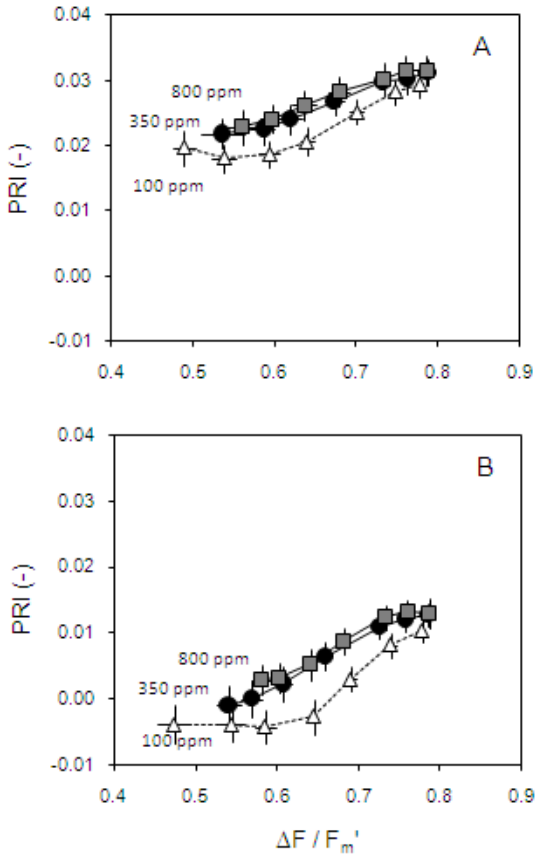

Fig. 11 Relationship between photochemical reflectance index (PRI) and PSII photochemical yield (Φ_{PSII}) in response to changes in irradiance (PAR) under three constant CO_2 concentrations in leaves of fertilized (a) and unfertilized (b) plants of *Populus euroamericana*.

The effect of leaf biochemical status on PRI had already been noted before (Garbulsky et al. 2011), commonly attributed to changes in chlorophyll content (Wu et al. 2010) or in carotenoid-to-chlorophyll ratios (Filella et al. 2009); (Garrity et al. 2011). This was indeed the case in the present study: the minimum PRI for each leaf is strongly related to REIP (Fig. 12;R^2= 0.85). REIP values change with changes in leaf chlorophyll concentration (Boochs et al. 1990); Horler et al., 1980; (Lamb et al. 2002), but also in the carotenoid/chlorophyll ratio (see Chapter 5) and as leaf nitrogen status is often related to chlorophyll content, the REIP has been used also as an indicator to estimate foliar nitrogen content indirectly (Cho and Skidmore 2006). Leaf xanthophyll content has been found to change substantially as a result of fertilization and in response to changes in leaf N content and photosynthetic potentials (Stylinski et al. 2000); (Cheng 2003),

similar changes in PRI are not surprising, as the index is related to the leaf content of zeaxanthin (Gamon and Surfus 1999).
So, we can say that PRI can also be a good indicator of the nutritional status of plants, and whether they need fertilization or not, as well as a short-term indicator to estimate leaf photosynthetic light use efficiency (Penuelas et al. 1995); (Gamon et al. 1997).

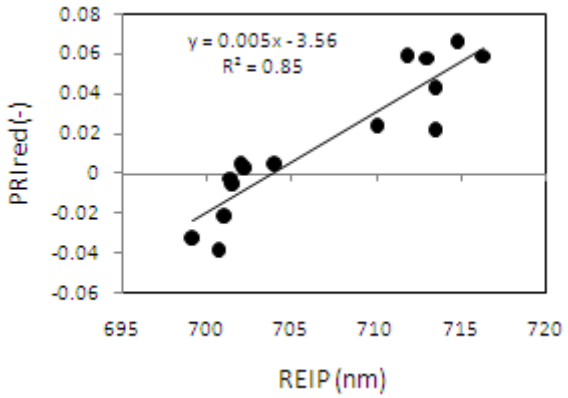

Fig. 12 Relationship between minimum values of photochemical reflectance index (PRI) and red edge index position (REIP) in leaves of fertilized (upper dots) and unfertilized (bottom dots) plants of *Populus euroamericana*.

4.5. Conclusions

The experimental test demonstrates a consistent pattern of co-variation between fluorescence and photochemical yield, whereas the Photochemical Reflectance Index (PRI) did not show a consistent relationship among treatments, being strongly affected by leaf nutritional status. As already suggested (Stylinski et al. 2000); (Filella et al. 2009); (Garrity et al. 2011), dark-acclimated PRI seems to be associated with leaf chlorophyll content (and hence REIP), or the ratio of carotenoids/ chlorophyll.

Ambient fluorescence could provide a useful tool for testing photosynthetic processes from a distance. However, there are still problems in its interpretation despite new models being proposed. This new understanding of ambient fluorescence opens novel perspectives for the airborne and satellite remote sensing of photosynthetic processes. That is because fluorescence showed to be

a reliable predictor of photosynthetic potentials of leaves once the effect of absorbance has been corrected for. This relationship is not a simple one, however, because it depends on whether photosynthesis is limited by light conditions (negative association) or CO_2 conditions (positive association).

Reference list

Boochs, F., Kupfer, G., Dockter, K., & Kuhbauch, W. (1990). Shape of the Red Edge As Vitality Indicator for Plants. *International Journal of Remote Sensing, 11*, 1741-1753

Cheng, L.L. (2003). Xanthophyll cycle pool size and composition in relation to the nitrogen content of apple leaves. *Journal of Experimental Botany, 54*, 385-393

Cho, M.A., & Skidmore, A.K. (2006). A new technique for extracting the red edge position from hyperspectral data: The linear extrapolation method. *Remote Sensing of Environment, 101*, 181-193

Evain, S., Flexas, J., & Moya, I. (2004). A new instrument for passive remote sensing. 2. Measurement of leaf and canopy reflectance changes at 531 nm and their relationship with photosynthesis and chlorophyll fluorescence. *Remote Sensing of Environment, 91*, 175-185

Filella, I., Porcar-Castell, A., Munne-Bosch, S., Back, J., Garbulsky, M.F., & Penuelas, J. (2009). PRI assessment of long-term changes in carotenoids/chlorophyll ratio and short-term changes in de-epoxidation state of the xanthophyll cycle. *International Journal of Remote Sensing, 30*, 4443-4455

Flexas, J., Escalona, J.M., Evain, S., Gulias, J., Moya, I., Osmond, C.B., & Medrano, H. (2002). Steady-state chlorophyll fluorescence (Fs) measurements as a tool to follow variations of net CO2 assimilation and stomatal conductance during water-stress in C-3 plants. *Physiologia Plantarum, 114*, 231-240

Franck, F., Juneau, P., & Popovic, R. (2002). Resolution of the Photosystem I and Photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature. *Biochimica Et Biophysica Acta-Bioenergetics, 1556*, 239-246

Gamon, J.A., Serrano, L., & Surfus, J.S. (1997). The photochemical reflectance index: an optical indicator of photosynthetic radiation use efficiency across species, functional types, and nutrient levels. *Oecologia, 112*, 492-501

Gamon, J.A., & Surfus, J.S. (1999). Assessing leaf pigment content and activity with a reflectometer. *New Phytologist, 143*, 105-117

Garbulsky, M.F., Penuelas, J., Gamon, J., Inoue, Y., & Filella, I. (2011). The photochemical reflectance index (PRI) and the remote sensing of leaf, canopy and ecosystem radiation use efficiencies A review and meta-analysis. *Remote Sensing of Environment, 115*, 281-297

Garrity, S.R., Eitel, J.U.H., & Vierling, L.A. (2011). Disentangling the relationships between plant pigments and the photochemical reflectance index reveals a new approach for remote estimation of carotenoid content. *Remote Sensing of Environment, 115*, 628-635

Genty, B., Briantais, J.M., & Baker, N.R. (1989). The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta, 990*, 87-92

Grace, J., Nichol, C., Disney, M., Lewis, P., Quaife, T., & Bowyer, P. (2007). Can we measure terrestrial photosynthesis from space directly, using spectral reflectance and fluorescence? *Global Change Biology, 13*, 1484-1497

Horler, D. N. H., Dockray, M., & Barber, J. (1983). The red edge of plant leaf reflectance. *International Journal of Remote Sensing, 4(2),* 273-288.

Lamb, D.W., Steyn-Ross, M., Schaare, P., Hanna, M.M., Silvester, W., & Steyn-Ross, A. (2002). Estimating leaf nitrogen concentration in ryegrass (Lolium spp.) pasture using the chlorophyll red-edge: theoretical modelling and experimental observations. *International Journal of Remote Sensing, 23*, 3619-3648

Louis, J., Ounis, A., Ducruet, J.M., Evain, S., Laurila, T., Thum, T., Aurela, M., Wingsle, G., Alonso, L., Pedros, R., & Moya, I. (2005). Remote sensing of sunlight-induced chlorophyll fluorescence and reflectance of Scots pine in the boreal forest during spring recovery. *Remote Sensing of Environment, 96*, 37-48

Malenovsky, Z., Mishra, K.B., Zemek, F., Rascher, U., & Nedbal, L. (2009). Scientific and technical challenges in remote sensing of plant canopy reflectance and fluorescence. *Journal of Experimental Botany, 60*, 2987-3004

Moya, I., Camenen, L., Evain, S., Goulas, Y., Cerovic, Z.G., Latouche, G., Flexas, J., & Ounis, A. (2004). A new instrument for passive remote sensing. 1. Measurements of sunlight-induced chlorophyll fluorescence. *Remote Sensing of Environment, 91*, 186-197

Penuelas, J., Filella, I., & Gamon, J.A. (1995). Assessment of Photosynthetic Radiation-Use Efficiency with Spectral Reflectance. *New Phytologist, 131*, 291-296

Pfundel, E. (1998). Estimating the contribution of Photosystem I to total leaf chlorophyll fluorescence. *Photosynthesis Research, 56*, 185-195

Schreiber, U., & Bilger W. (1987). Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. In: *Plant Response to Stress.Functional Analysis in Mediterranean Ecosystems*, edited by Tenhunen, J. D., Catarino, F. M., Lange, O. L., & Oechel, W. C., Berlin:*Springer-Verlag,* 27-53

Stylinski, C.D., Oechel, W.C., Gamon, J.A., Tissue, D.T., Miglietta, F., & Raschi, A. (2000). Effects of lifelong CO2 enrichment on carboxylation and light utilization of Quercus pubescens Willd. examined with gas exchange, biochemistry and optical techniques. *Plant Cell and Environment, 23*, 1353-1362

Wu, C., Niu, Z., Tang, Q., & Huang, W. (2010). Revised photochemical reflectance index (PRI) for predicting light use efficiency of wheat in a growth cycle: validation and comparison. *International Journal of Remote Sensing, 31*, 2911-2924

Chapter 5

Reflectance indices for the assessment of photosynthetic pigments content and nutritional status of poplar trees

5.1. Abstract

Plant nutritional status and its effects on plant growth is commonly monitored through destructive sampling followed by chemical analysis in the laboratory, which is expensive and time consuming. Instead, there are other methods to measure the nutrient status of plants indirectly. These methods, based on the proximal remote-sensing of leaf reflectance using new spectroscopic techniques, are less expensive, are time saving and easy to use than traditional wet-chemistry analyses. Among the most widely applied techniques, we tested the SPAD-502 chlorophyll meter and a number of spectral indexes derived from leaf reflectance, as measured by the LICOR Li-1800 Spectroradiometer. The SPAD is a simple, portable diagnostic tool that measures the greenness or the relative chlorophyll content of leaves. The LICOR Li-1800 measures the reflectance and transmittance of the leaf within the 400-1100 nm range, from which a number of reflectance indices such as REIP, PRI, SIPI and PSRI can be then estimated for the estimation of leaf biochemistry. These indices use particular wavelengths of the reflectance spectra which contain information about the photosynthetic pigments content of leaves. The objective of this study was to estimate the effects of fertilization and nutrient availability on growth of *Populus x euroamericana* saplings and to develop new methods to assess the need for fertilization, based on the remote sensing of vegetation pigments. Five months-old saplings of poplar (*Populus euramericana*) clone AF2, which is a species of interest in forestry, were used for the measurements of SPAD and LICOR Li-1800. Some spectral reflectance indices proposed in the literature were tested in

order to determine the best one for the detection of changes in leaf photosynthetic pigment content. These novel techniques are of high relevance for applied research, as they could provide a fast and reliable method to estimate these biochemical parameters in the field, and to estimate the nutrient requirements of our plants for optimal growth.

5.2. Introduction

Fertilization and nutrient availability have a large effect on the growth and development of plants, largely as a result of changes in chlorophyll and nitrogen (N) content, which together determine the photosynthetic capacity of leaves. So, it is very important to manage the nutritional processes in crop cultivation, supplying adequate quantities of N to crops in order to optimize crop yield. While a lack of nutrients can reduce the profitability of cultivation, however, excessive N application can contaminate water resources by leaching and run-off of the excess of N from soil (Jaynes et al. 2001). Therefore it is important to optimize N fertilization through the efficient monitoring of plant N status and an appropriate management of N fertilizer which will minimize the perturbations of the environment (Jaynes et al. 2001).

An indirect measure of nutrient status can be achieved by quantifying leaf chlorophyll content (Filella et al. 1995; Moran et al. 2000; Richardson et al. 2002), making the precise measurement of leaf pigmentation important to both land managers and ecophysiologists.. A close correlation often exists between leaf chlorophyll and nitrogen concentrations (Boochs et al. 1990; Everitt et al. 1985; Yoder and Pettigrewcrosby 1995), as N is part of the chemical structure of chlorophyll molecule and a key component of proteins associated to chlorophyll in light-harvesting complexes. Moreover, the foliar concentration of photosynthetic pigments is a parameter of interest in itself, because of its strong effect on the amount of solar radiation absorbed by a leaf. The resulting excitation will be channeled to pigments' reaction centers leading to electron release and begin the photochemical process. The most important of these pigments are chlorophylls, which are essential for the oxygenic conversion of light energy to the stored chemical energy (Richardson et al. 2002). Therefore, low

chlorophyll concentrations will reflect in reduced light absorption and photosynthesis, reflecting in lower primary production at canopy scale (Curran et al. 1990; Filella et al. 1995). As a result, changes in the levels of chlorophylls and carotenoids and in the ratio of chlorophyll a to chlorophyll b in foliage have been used to evaluate photosynthetic activity, have been often used as an indicator of leaf function and abiotic stress in plants.

The assessment of leaf chlorophyll and nitrogen contents is generally based on destructive wet chemistry techniques, through the extraction of photosynthetic pigments from excised plant materials using organic solvents, followed by an HPLC or spectrophotometric assessment of pigment content on leaf extracts. Beside sample destruction, these methods may lead to a high variability in the results, because of pigment losses during the extraction and dilution processes. In addition, they are expensive and time consuming, making the assessment of vegetation health and nutrient requirements impractical at ecosystem or landscape level.

Other methods have been proposed, however, for the estimation of photosynthetic pigments, based on dedicated chlorophyll meters (such as the Minolta SPAD-502), or on the measurement of leaf spectral reflectance and the assessment of indices such as the red-edge index position (REIP; Horler et al. 1983). These methods are nondestructive, fast and reliable, can be used to estimate plant nutritional status directly in the field, resulting in an immediate assessment of whether or not any fertilization is neededfor the management of crop cultivation systemsSome of these techniques could even be applied across spatial scales through the application of airborne or satellite platforms (Gamon and Qiu 1999).

As an example, the chlorophyll meter (SPAD-502; Minolta Camera Co. Ltd.) is a simple, portable diagnostic tool that measures the greenness or the relative chlorophyll concentration of leaves (Inada 1985). The device was developed to make fast (2s) and non-destructive readings on a leaf based on the measurement of leaf transmittance in the red spectral region at about 650 nm, where the light emitted by a diode is strongly absorbed bychlorophylls. A second wavelength at about 940 nm, in the infrared region, where no light absorption occurs, is used as a reference and to compensate for leaf thickness. Based on the alternative

transmittance at these wavelengths, an arbitrary index is calculated, which can be translated into quantitative measurements of leaf chlorophyll content through a careful calibration against wet chemistry measurements (Netto et al. 2005). Several studies have established the relationship between SPAD-502 readings and total chlorophyll concentration for several plant species (Marquard and Tipton 1987; Schaper and Chacko 1991) reporting simple linear mathematical model fitted in these studies. Some other studies correlate SPAD-502 readings with nitrogen content in several plant species (Smeal and Zhang 1994; Peng et al. 1995) as this element is part of the chemical structure of the chlorophyll molecule, and that makes it a promising tool for improving N management.
It is a practical device as it saves time, space and resources compared with the traditional destructive methods (Netto et al. 2005). Thus, the portable chlorophyll meter (SPAD-502) can help in the advanced interpretations of photochemical process in plants (Netto et al. 2005). The instrument has been applied for the assessment of leaf biochemical contents and fertility in a range of tree species (see review below). However no information is available on the use of this meter for poplar hybrids commonly used throughout Europe, which are of particular interest in plantation and short-rotation forestry.

Spectral reflectance measurements of fresh leaves in the visible and red-edge wavelengths (400–700 nm) have also been used to determine foliar chlorophyll and nitrogen concentration (Bausch and Duke 1996; Sullivan et al. 2004). Variations in leaf chlorophyll content have been detected through changes in spectral reflectance and related to leaf development and senescence (Carter and Knapp 2001; Gamon and Surfus 1999; Gitelson and Merzlyak 1994a, b) and soil fertility (Carter and Knapp 2001; Chappelle et al. 1992; Mariotti et al. 1996; McMurtrey et al. 1994; Yoder and Pettigrewcrosby 1995).
Fresh leaf lignin and nitrogen concentrations, on the contrary, are commonly found to be best predicted from near- and short-wave infrared reflectance features (Peterson et al. 1988), although with a predictive power lower than with dried, ground material. Any correlation with visible reflectance features are likely to be the result of the close association between nitrogen and chlorophyll pigments (Haboudane et al. 2004; Hansen and Schjoerring 2003; Yoder and Pettigrewcrosby 1995) because pigments (chlorophyll, carotenoids and

xanthophylls) predominantly determine most spectral features between 400 and 700 nm (Blackburn 1998; Carter and Knapp 2001; Woolley 1971; Yoder and Pettigrewcrosby 1995). Because of this association, the reflectance assessment of chlorophyll content can be used to characterize the nitrogen status of vegetation and crops (Filella and Penuelas 1994), and several studies have observed a decrease in leaf absorbance and a corresponding increase in reflectance and transmittance in the visible wavelengths in response to nutritional deficiencies (Chappelle et al. 1992; McMurtrey et al. 1994).

In particular, the red-edge index position (REIP) has been proposed as an optimal tool for evaluating the changes in spectral properties, as the region of the red-near infrared (NIR) transition has been shown to have the highest information content for vegetation spectra (Collins, 1978; Horler et al., 1983). The red-edge represents the region of abrupt change in leaf reflectance spectra between 680 and 780 nm caused by the combined effects of strong chlorophyll absorption in the red wavelengths and high reflectance in the NIR wavelengths due to leaf internal scattering (Horler et al., 1983). When the amount of chlorophyll increases, a broadening of the major chlorophyll absorption feature centered around 680 nm is observed (Buschmann and Nagel 1993; Dawson and Curran 1998), causing a shift in the red edge slope and wavelength of maximum slope (or inflection point, corresponding to REIP) towards longer wavelengths (Boochs et al. 1990; Clevers et al. 2002; Horler et al. 1980, 1983).
Horler et al. (1983) first examined the feasibility of using the REIP of leaf reflectance spectra as an indication of plant chlorophyll status; a high positive correlation was found in all species examined between REIP and leaf chlorophyll content, although there were some differences in the quantitative nature of the relationship for plants of different types.
Several other experimental studies have since demonstrated that low leaf chlorophyll concentrations are associated with REIP values near 700 nm, while high chlorophyll concentrations in combination with a high leaf internal scattering induce REIP values of up to 725 nm (Boochs et al. 1990; Horler et al. 1980; Lamb et al. 2002). (Sims and Gamon 2002) also found that an index based on the first derivative of reflectance in the red edge region was insensitive to leaf structural variation. In addition, the presence of other pigments did not

significantly affect estimation of chlorophyll from spectral reflectance. As a result, REIP has been used as a reliable indicator of chlorophyll content at the leaf level (Horler et al., 1983; (Curran et al. 1991; Curran et al. 1995; Filella and Penuelas 1994; Clevers et al. 2002; Lamb et al. 2002; Smith et al. 2004), and also (albeit more controversially) at canopy level (Filella and Penuelas 1994). Since a sizeable fraction of leaf nitrogen is associated with chlorophyll molecules and with associated proteins in light-harvesting complexes, the REIP has been used as a means to estimate foliar nitrogen content indirectly (Cho and Skidmore 2006; Lamb et al. 2002).

Carotenoids are very important pigments, involved in protecting the photosynthetic apparatus against photodamage (Demmigadams 1990). When the energy of the incident light exceeds what is needed for photosynthesis, leaf carotenoids (and in particular leaf xanthophylls) dissipate excess harmful energy avoiding damage to the photosynthetic system (Demmigadams and Adams 1996; Ort 2001).

The higher concentration of chlorophyll than carotenoids in most leaves, and the overlap between chlorophyll and carotenoids absorption peaks, make it much more difficult the estimation of leaf carotenoids content from reflectance than the estimation of chlorophyll. Consequently, the carotenoids-to-chlorophyll ratio was estimated more successfully through reflectance indices than absolute carotenoids contents (Merzlyak et al. 1999; Penuelas et al. 1995). The ratio between chlorophyll and carotenoids may be a good indicator for distinguishing the natural autumn senescence from senescence due to natural stress such as desiccation in mosses (Buckland et al. 1991), and drought in flowering plants (Seel et al. 1992). Carotenoids/chlorophyll ratio increases in stress conditions or during leaf senescence because chlorophyll decreases more rapidly than carotenoids (Gitelson and Merzlyak 1994b; Penuelas et al. 1995). So, variation in this ratio can be a good marker of stress in plants.

Most of the reflectance indices proposed for the estimation of the carotenoid-to-chlorophyll ratio are based on the comparison of reflectance in wavelengths of the carotenoids absorption peak (400-500 nm) with reflectance in the red region, which is influenced only by chlorophyll. The “structure-insensitive pigment index”

(SIPI) was developed by (Penuelas et al. 1995),while the “plant senescence reflectance index” (PSRI) was originally developed by (Merzlyak et al. 1999). The “photochemical reflectance index” (PRI), originally proposed to estimate rapid changes in the relative levels of pigments of the xanthophylls cycle and in non-photochemical energy dissipation (Gamon et al. 1992), has also been found to be correlated in a general relationship with the carotenoids/chlorophyll ratio (Sims and Gamon 2002).

The objective of this study is to estimate the effects of fertilization and nutrient availability on plant growth and leaf biochemistry in young poplar (Populus x euroamericana) trees, and to evaluate the use of remote sensing techniques for the assessment of leaf pigments and fertilization requirementsRemote sensing techniques have been used to estimate different parameters of leaf biochemistry (nitrogen, chlorophyll, carotenoids), as well as leaf absorbance and reflectance. They could provide a fast and reliable method to estimate these parameters in the field, and therefore to evaluate the nutrient requirements of our plants for optimal growth. The study has therefore important practical applications for the management of poplar cultivation systems.

5.3. Material and methods

5.3.1. Plant material

Twenty hardwood cuttings of poplar (*Populus euramericana*) clone AF2 were planted on March 19, 2010 in pots of 11.3 L capacity containing a mixture of sand and peat (1:1, by vol.). The pots were maintained in “Cadriano”, the experimental field of the faculty of agriculture/ university of Bologna with adequate watering supply. Ten pots were fertilized with 100 kg/ha of an agricultural inorganic NPK fertilizer (containing 21% NO3-N, 7% P2O5-P, and 14% K2O-K).

Ten other plots did not receive any fertilizer and were used as a control.

At the end of the experiment, when plants were five months old, detailed measurements of height, stem base diameter, number of leaves, and the dimensions of twenty leaves per plant were done. An allometric relationship between leaf dimensions and leaf area (Barigah et al. 1994) was applied to

calculate the area of individual leaves for the twenty leaves chosen, and then the total leaf area per plant was calculated multiplying the mean leaf area by the number of leaves. Based on a relation developed in a parallel study on 1-year-old plants of the same clone, plant biomass was calculated (E. Muzzi, pers. comm.)

5.3.2. SPAD-502 readings

The portable chlorophyll meter (SPAD Minolta 502, Minolta LTD., Osaka, Japan) was used to non-destructively measure leaf chlorophyll (Chl a+b) content. SPAD has a 0.06-cm^2 measurement area, and calculates an index in 'SPAD units' based on absorbance at 650 and 940 nm. The accuracy of the SPAD is claimed to be 1.0 SPAD units. The mean of five readings was obtained for each of 60 healthy fully expanded mature leaves in 8 different plants in order to explore a range from the minimum chlorophyll content readings to the maximum. Thirty of these leave were chosen in a gradation of SPAD readings from 23 to 49. During measurements with SPAD 502, the sensor head was shaded to avoid direct sunlight from reaching the instrument.

5.3.3. LI-COR Li-1800 Spectroradiometer measurements

The LICOR LI-1800 (LI-COR, inc., Lincoln, Nebraska, USA) hyperspectral spectroradiometer provided with the 1800-12 external integrating sphere was used to measure the reflectance and the transmittance of the 30 leaves in a wavelength range between 400 and 1100 nm, and on a spot of diameter 1.14 cm, avoiding the major veins. The instrument has a sampling frequency of about 2 nm, and a FWHM spectral resolution of about 3 nm, making it suitable for the assessment of REIP and of selected spectral indices. The data obtained were being used to calculate the absorbance of these leaves.

Measurements were carried out at the laboratory of ecophysiology in the Faculty of Agriculture of the University of Bologna.

Use of the integrating sphere made it possible to evaluate total leaf absorbance and hemispherical spectral reflectance, from which several indexes could be evaluated. After applying a Savitzky-Golay smoothing filter, red-edge index

position (REIP) was computed as the wavelength corresponding to the highest first difference in reflectance Horler *et al.* (1983).

The “structure-insensitive pigment index” (SIPI; (Penuelas et al. 1995), the “plant senescence reflectance index” (PSRI; (Merzlyak et al. 1999) and the “photochemical reflectance index” (PRI; (Gamon et al. 1992) were computed as:

SIPI = $(R_{800} – R_{445}) / (R_{800} – R_{680})$

PSRI = $(R_{680} – R_{500}) / R_{750}$

PRI = $(R_{531} – R_{570}) / (R_{531} + R_{570})$

where Ri is leaf hemispherical reflectance at wavelength i.

Total leaf absorbance in the visible range (400-700 nm) was also estimated from spectroradiometric measurements, as the raw average of absorbance at individual wavelengths within this interval.

5.3.4. Chlorophyll and carotenoids content measurements

The extraction of the photosynthetic pigments chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoid (Car) were carried out as described by (Minocha et al. 2009). In short, two disks of 2 cm diameter each were taken from each leaf immediately after the measurements carried on with the spectroradiometer and from the same place which had been exposed to measurements. One of them was dried at 65 °C for 48 h to be used for the N content assessment, whilst the other was placed in a 2 mL microfuge tubes (Eppendorf Safe Lock, Eppendorf North America, Westbury, New York) and stored at –20 °C until analysis. At the time of extraction 6 mL of the solvent *N,N*-dimethylformamide (DMF) were added to 60 mg of the plant tissue in glass tubes with Teflon cap, and incubated in the dark at room temperature (25 °C) for 24 h. A UV-Visible Spectrophotometer CARY 1E (Varian-Agilent Technologies, Santa Clara, CA, USA) was used to record absorbances in the range of 450 to 700 nm using 0.7 mL aliquots in quartz microcuvettes (Quartz Suprasil, Hellma Cells Inc., Plainview, New York).

Total chlorophyll, chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) were calculated according to the equation proposed by Lichtenthaler and Wellburn (1983) for 1-4 nm spectrophotometer resolution range.

5.3.5. Nitrogen content assessment

After drying as described above, samples were put in microfuge tubes and milled using the mixer mill Retsch MM 300 (Retsch GmbH and Co. KG, Haan, Germany). About 1.5 mg from each milled sample was used for the analysis of nitrogen content and concentration using an HCL elemental analyzer HCL (EA 1110, Carlo Erba instruments, Milan, Italy).

5.4. Results and discussion

5.4.1. Effects of fertilization on plant growth and characteristics

Fertilization and nutrient availability had a large effect on the growth of poplar cuttings, largely as a result of the changes in chlorophyll and nitrogen content. Data of average growth and biometry, as well as foliar biochemical contents, for the two treatments are presented in Table 1.

From Table 1 we can see that there is a significant difference between the fertilized and unfertilized plants in plant height. Fertilized plants also had much longer and wider leaves (and therefore a bigger area of individual leaves). Fertilized plants also have a much greater leaf area per plant. Diameter at the base of the stem on the contrary was not so much different between treatments. We could also estimate plant biomass as a function of diameter at the base and plant height, and a significance difference was observed as a result of fertilization. As expected, leaf chlorophyll content in fertilized plants was also significantly higher than in the unfertilized ones; the same can be said for carotenoid content and leaf N concentration. As a result of the difference in leaf pigments, total PAR absorbance in the 450-700 nm range was also higher in the fertilized treatment.

Table 1 Effects of fertilization on biometric and biochemical characteristics of 6 months old cuttings of *P. euroamericana.* The table presents average values ± SE.

	Fertilized	**Unfertilized**
Height (cm)	149.325 ± 4.96	131.1 ± 2.63
Diameter (cm)	0.9 ± 0.00	0.8 ± 0.05
N° of Leaves	41.5 ± 1.73	40 ± 1.05
Leaf length (cm)	11.35 ± 0.18	9.47 ± 0.12
Leaf width (cm)	10.62 ± 0.11	8.72 ± 0.11
Leaf area (cm2)	81.49 ± 1.94	55.79 ± 1.36
Total leaf area (cm2/plant)	3382.9245 ± 161.39	2237.03 ± 179.59
Biomass (g DM/plant)	13.40 ± 0.00	9.69 ± 1.66
Chl(a+b) (mg/cm2)	0.0344 ± 0.0018	0.0182 ± 0.0008
PAR absorbance (%)	84.39 ± 0.5286	77.24 ± 0.6182
SPAD reading (a.u.)	40.41 ± 1.12	27.27 ± 0.807
REIP (nm)	710.12 ± 1.16	701.28 ± 0.4547
Carotenoids (mg m-2)	61.63 ± 2.06	41.42 ± 1.19
N content (g N/m2)	1.23 ± 0.06	0.85 ± 0.03
N concentration (g N/g DM)	0.00039 ± 0.00002	0.00027 ± 0.000009

So, we can stress that fertilization had a significant effect on growth, so demonstrating why it is so important to be able to estimate the effects of fertilization on leaf biochemistry, and the nutrient requirements of poplar trees. These results are of considerable importance for the practical management of poplar cultivation, in order not to waste fertilizer if not needed, and not to risk a loss of productivity on the contrary if there was a need for fertilization.

5.4.2. Effects on absorbance

The reflectance and absorbance between 400 and 1100 nm of a typical fertilized and an un-fertilized plant, as estimated by the LiCor-1800 provided with the integrating sphere, are presented in Fig. 1. We see in the graph to what extent the spectra differ as a result of differences in chlorophyll (and carotenoid) contents induced by fertilization. Also highlighted in the graph are the two wavelengths used in the SPAD instrument for the assessment of leaf chlorophyll content. And as the SPAD reading is based on absorbance measurement at two different wavelengths, so we can understand the difference in SPAD readings.

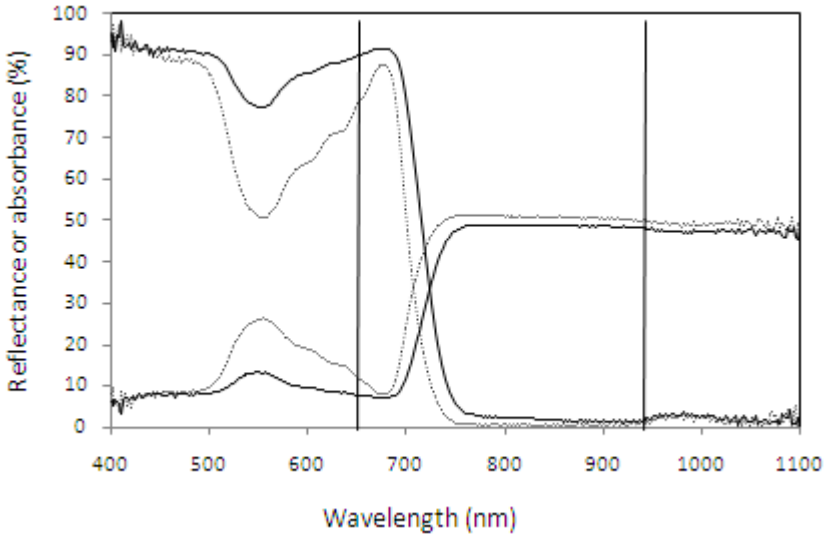

Fig. 1 The reflectance (bottom curves) and the transmittance (upper curves) of two *P. euroamericana* leaves with the highest (leaf #1; continuous line), and the lowest chlorophyll content (leaf #30; dotted line). Vertical bars show the two wavelengths (650 and 940 nm) used by SPAD for making instantaneous and non-destructive readings of leaf greenness or relative chlorophyll content.

5.4.3. Chlorophyll and reflectance at different wavelengths

In order to better understand changes in REIP readings and reflectance indices, it is instructive to look at changes in reflectance at the different wavelengths as a function of total chlorophyll (Chl a+b) content (Fig. 2). In the blue (444 nm) and the red domain (680 nm), where chlorophyll absorbs very strongly, absorbance and reflectance are already saturated at rather low values of chlorophyll content (150 mg m^{-2}), so that the signal at these wavelengths is not correlated with variable chlorophyll content (Fig. 2A and D). We see the same weak correlation with the reflectance in the near-infrared (940 nm: a wavelength used as a reference by the SPAD) where reflectance is a function of leaf structure, rather than chlorophyll, and therefore doesn't change (or changes very little) with leaf biochemical content (Fig. 2F).

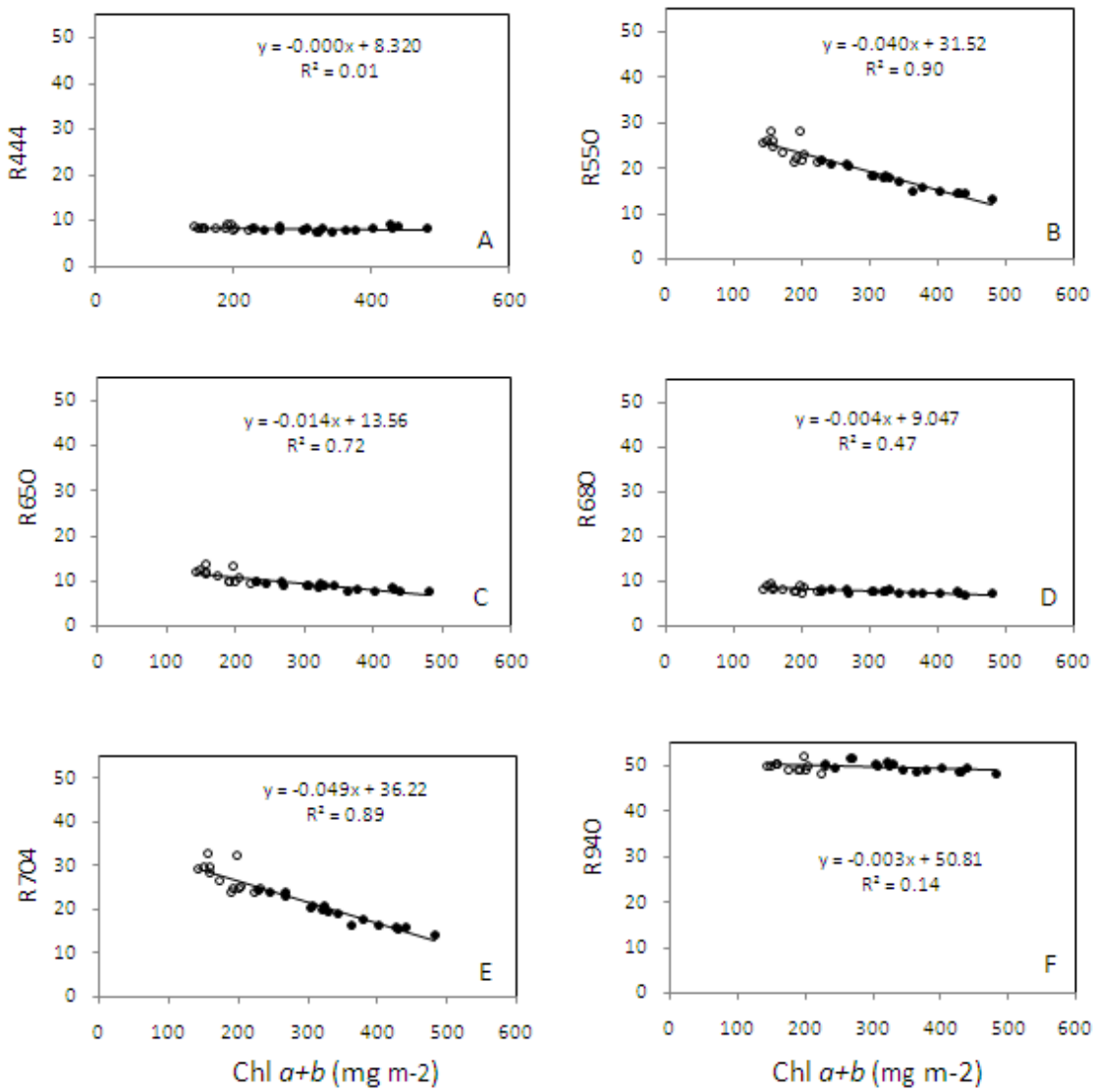

Fig. 2 Reflectance at different wavelengths as a function of total chlorophyll (Chl *a+b*) content for *P. euroamericana* leaves. Results for fertilized (●) and unfertilized (○) plants are presented.

On the contrary we see some changes in the reflectance at 650 nm (the true signal wavelength used by the SPAD instrument), which explains the observed changes in the SPAD readings with leaves of different chlorophyll content (Fig. 2C). As expected, chlorophyll content was best correlated with the reflectance at 550 nm (in the green; Fig. 2B), and at 704 nm (close to the red-edge position; (Fig. 2E).

5.4.4. Non-destructive estimation of leaf chlorophyll content

Leaf chlorophyll content was estimated by two non-destructive methods, using the SPAD instrument and from leaf reflectance through the computation of the red-edge index position (REIP).

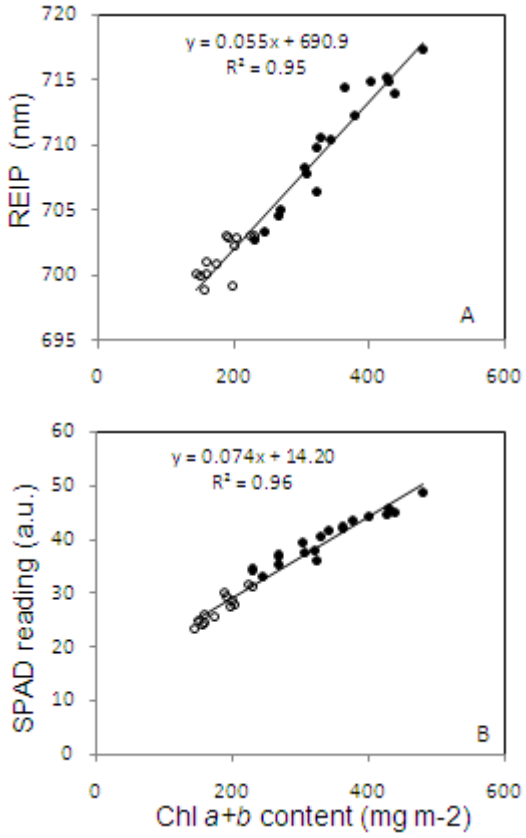

Fig. 3 Relationship between total chlorophyll (Chl *a* + *b*) content and the red-edge index position (REIP) (A) or the SPAD readings (B) in *P. euroamericana* leaves. Results for fertilized (●) and unfertilized (○) plants are presented.

The correlation between the red-edge position - the wavelength of the maximum slope in the increase of the reflectance from red to near infrared - estimated by the Licor-1800 spectroradiometer and total leaf chlorophyll content was very good as expected (R^2=0.95; Fig.3A). An even stronger linear relationship was observed between SPAD readings and the total chlorophyll content (Fig. 3B), with an R^2=0.96. In both cases, however, the relationship cannot be safely extrapolated from fertilized treatments to unfertilized ones or vice versa, as the associated error would be rather substantial. In the case of REIP, the regression line for the entire population is y = 0.055x + 690.9 with an R^2 = 0.95, while it is y =

0.06x + 689.23 (R^2 = 0.92) for fertilized plants, and y = 0.040x + 693.9 with an R^2 = 0.52 for unfertilized ones. We can say the same about the relationship between SPAD readings and total chlorophyll content: the regression line for the entire population is y = 0.074x + 14.20 with an R^2 = 0.96, while it is y = 0.06x + 20.13 with an R^2 = 0.93 for fertilized plants, and y= 0.091x + 10.66 with an R^2 = 0.85 for unfertilized ones. The need for a detailed calibration of both methods over as wide an interval as possible is clearly apparent.

5.4.2. *SPAD as a predictor of chlorophyll a,b and carotenoids*

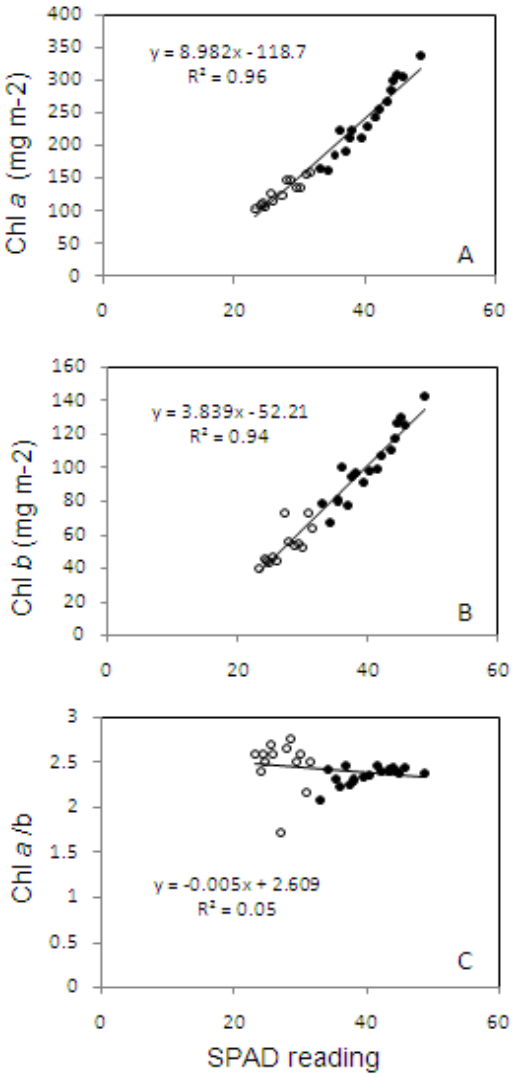

Fig. 4 Relationships between the SPAD-502 readings and chlorophyll *a* (Chl *a*) (A), chlorophyll *b* (Chl *b*) (B) and chlorophyll *a/b* (Chl *a/b*) (C) in *P. euroamericana* leaves. Results for fertilized (●) and unfertilized (○) plants are presented.

A strong relationship was also observed between SPAD readings and both chlorophyll a (Fig. 4A) and chlorophyll b contents (Fig. 4B), but there was no significant relationship with the ratio between them (chlorophyll a/b) (Fig. 4C). We found also a strong relationship between SPAD readings and total carotenoid content ($y = 1.548x - 0.877$; $R^2 = 0.94$). So, we can say that SPAD readings are a good predictor of chlorophyll a, chlorophyll b and carotenoids. The same is true for REIP, for which the regression line was $y = 0.723x + 681.1$, with an $R^2 = 0.93$ (data not shown).

5.4.5. Estimation of leaf absorbance

Photosynthetic pigments (mainly chlorophylls and carotenoids) absorb in the visible spectrum (400 – 700 nm), so that leaf reflectance is typically low in this domain (Penuelas and Filella 1998), and declines with increasing pigment content; leaf absorbance will of course increase in a specular way.

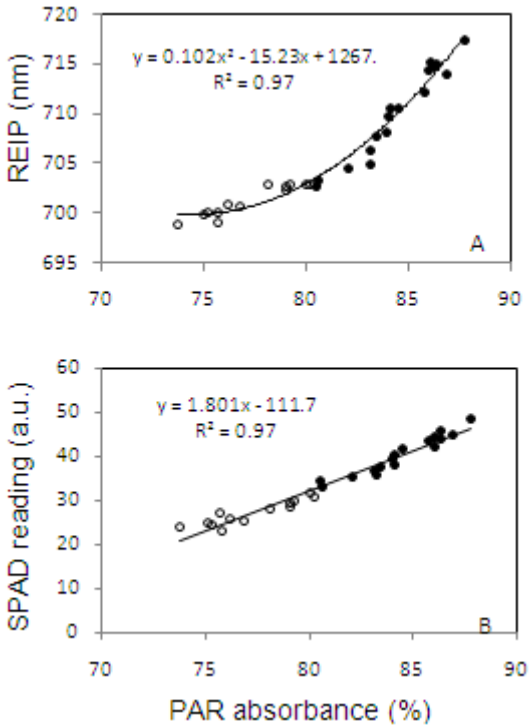

Fig. 5 Relationship between the PAR absorbance (%) and the red-edge index position (REIP) (A) or SPAD readings (B) in *P. euroamericana* leaves. Results for fertilized (●) and unfertilized (○) plants are presented.

As expected, the red-edge position was found to be a good predictor of leaf absorbance in the visible range (Fig. 5A). The relationship between PAR absorbance and the red-edge index position (REIP) is consistently curvilinear (second order polynomial; R^2=0.97). The relationship between SPAD readings and leaf absorbance, on the contrary, was linear (Fig. 5B) with an R^2 = 0.97. But as we saw before, it is not possible to extrapolate these relationships from fertilized to unfertilized treatments or vice versa. That is because in the case of the relationship between REIP and PAR absorbance, the best-fit regression line for the entire population is $y = 0.102 x^2 - 15.23 x + 1267$ ($R^2 = 0.97$), while it is $y = 2.132 x + 530.1$ ($R^2 = 0.94$) for fertilized plants, and $y = 0.698 x + 647.3$ ($R^2 = 0.90$) for unfertilized ones, with an obvious difference between the two curves. The same can be said about the relationship between SPAD readings and PAR absorbance: the regression line for the entire population was $y = 1.801 x - 111.7$ ($R^2 = 0.97$), while it is $y = 2.054 x - 132.9$ ($R^2 = 0.94$) for fertilized plants and $y = 1.210 x - 66.21$ ($R^2 = 0.86$) for unfertilized ones.

5.4.6. Chlorophyll and carotenoids

As apparent from Fig. 6, there is a very tight relationship between carotenoids and chlorophyll in sampled poplar leaves. So it is not surprising that any index able to predict chlorophyll, will be able to predict carotenoids as well. As we could estimate the chlorophyll using both the SPAD and the reflectance with the method of the red-edge position, we could also estimate the carotenoids using the same methods. A stronger relationship was observed between SPAD readings and carotenoids content ($y = 1.548 x - 0.877$; $R^2 = 0.94$), than between REIP and carotenoids content ($y = 2.018 x – 1372$; $R^2 = 0.90$).
Because of the photoprotective role of carotenoids, however, a more interesting question is whether any of these indices is able to predict the ratio between carotenoids and chlorophyll.

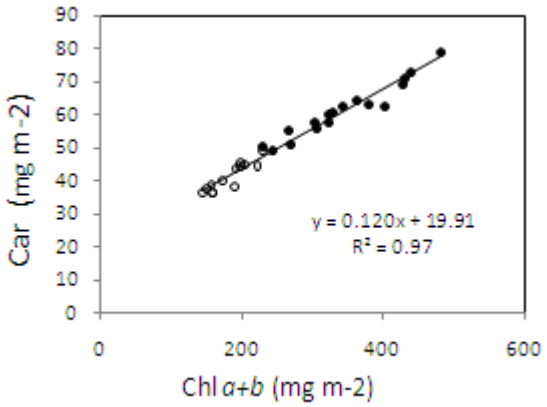

Fig. 6 Relationships between total chlorophyll (Chl *a*+*b*) and carotenoids (Car), in *P. euroamericana* leaves. Results for fertilized (●) and unfertilized (○) plants are presented.

5.4.7. Reflectance indices

In 2002 Sims and Gamon reviewed several indices to estimate pigment content based on leaf reflectance at particular wavelengths, such as the "structure-insensetive pigment index" (SIPI), which was developed by (Penuelas et al. 1995), the "plant senescence reflectance index" (PSRI) which was developed by (Merzlyak et al. 1999), and the "photochemical reflectance index" (PRI) which was originally developed by (Gamon et al. 1992) to estimate rapid changes in the relative levels of pigments of the xanthophylls cycle. They found that PRI was best performing with a significant correlation with the carotenoid/chlorophyll ratio (R^2 = 0.39), but neither SIPI nor PSRI were significantly correlated with the pigment ratio. In our study of poplar response to fertilization, the relationship between PRI and the carotenoids/chlorophyll ratio was even better (Fig. 7B) with an R^2 = 0.73. The SIPI was also found to perform reasonably well (Fig. 7A) with an R^2 = 0.5, whereas PRSI was poorly correlated with the pigment ratio (Fig. 7C). The relationship between PRI and pigment ratios confirms previous reports (Filella et al. 2009; Garrity et al. 2011) and is of particular interest. The Photochemical Reflectance Index was originally proposed for the estimation of xanthophylls interconversion and photosynthetic light use efficiency, and such a relationship has been confirmed at the leaf (Gamon et al. 1997; Penuelas et al. 1995; Penuelas et al. 1997), canopy (Filella et al. 1996; Gamon et al. 1992;

Trotter et al. 2002), and ecosystem scale (Nichol et al. 2000; Penuelas and Inoue 2000). As a result, PRI has been found to track both daily (Gamon et al. 1992; Penuelas et al. 1994) and seasonal (Filella et al. 2009; Garbulsky et al. 2008; Stylinski et al. 2002) variation in photosynthetic activity. A re-analysis of the literature (Garbulsky et al. 2011), however, suggests that whilst over the short term the variation of PRI will primarily be a function of changes in xanthophylls de-epoxydation state and light-use efficiency, variation of PRI over weeks or months may be affected mainly by changes in the total pools of xanthophylls, carotenoids and chlorophylls (Gamon et al. 2001).

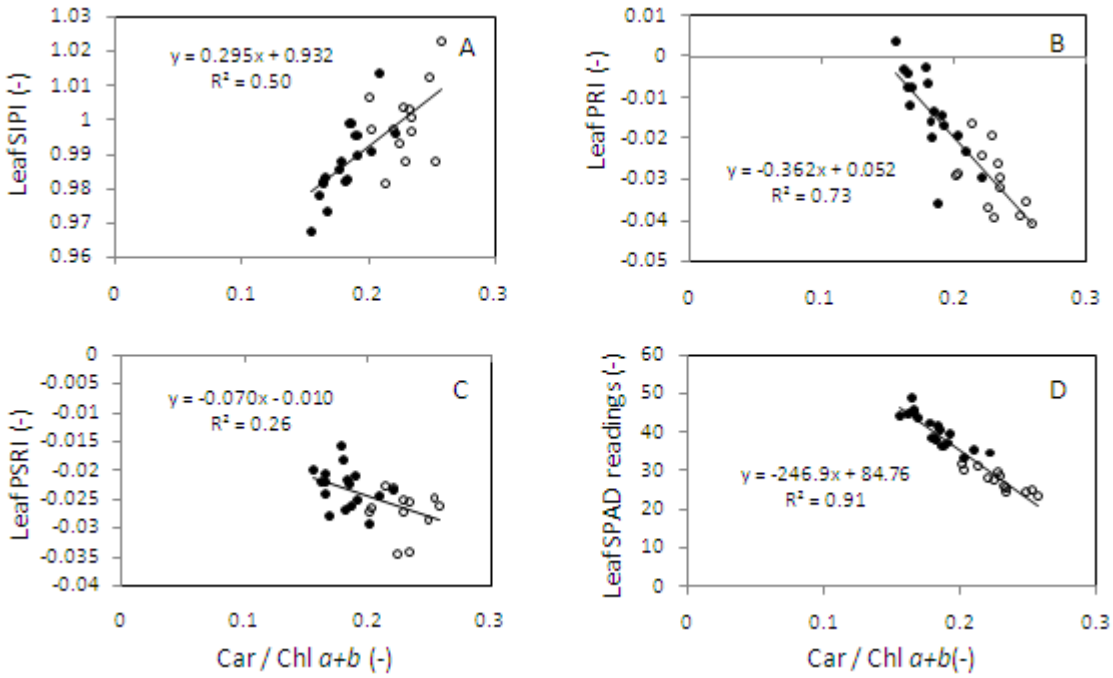

Fig. 7 The SIPI (Penũelas et al., 1995) (A), PRI (Gamon et al., 1992) (B), PSRI (Merzlyak at al., 1999) (C), and the SPAD (D) as functions of leaf carotenoids to total chlorophyll ratio (Car/Chl *a+b*) for thirty leaves of *P. euroamericana* of gradient SPAD readings. Results for fertilized (●) and unfertilized (○) plants are presented.

It should be mentioned, however, that even better results were obtained with another index proposed by (Sims and Gamon 2002), the mND705, which showed a tight inverse relationship with carotenoid/chlorophyll ratios ($y = -4.102 x + 1.330$; $R^2 = 0.89$). As for the SPAD, it was also found to be a very good tool to estimate the pigment ratio with a significant inversely correlation (Fig. 7D) with an

R^2 = 0.91. This is of particular practical relevance, for the simplicity of the technique, which does not require a spectrometer. The REIP was also found to correlate significantly with the carotenoids/chlorophyll ratio (y = -179.6 x + 742.6; R^2 = 0.85).

5.4.8. Estimation of leaf nitrogen (N) (concentration and content):

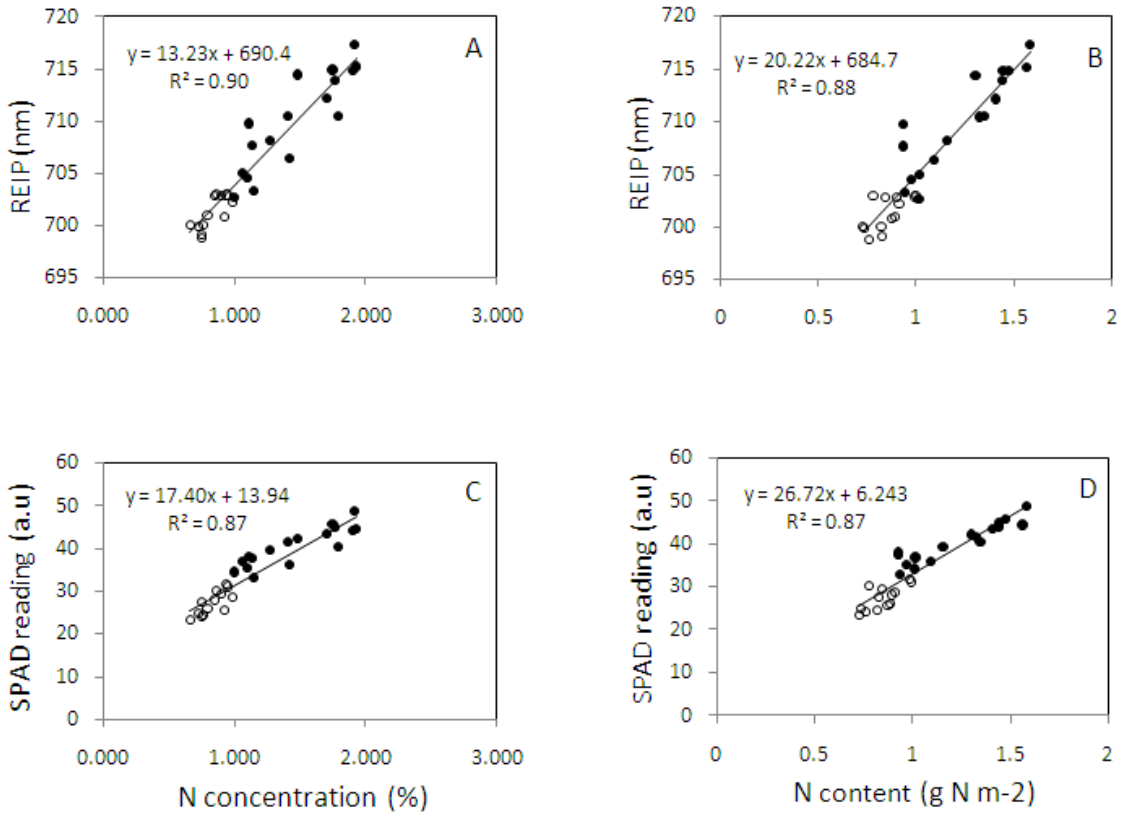

Fig. 8 Relationship between N concentration or N content and the red-edge index position (REIP) (A, B) or the SPAD readings (C, D) in *P. euroamericana* leaves. Results for fertilized (●) and unfertilized (○) plants are presented.

The potential of spectroscopic techniques for the assessment of leaf N status was also tested. It would be expected that nitrogen content behave better than N concentration, as leaf reflectance should be related to the amount of pigments per unit leaf area; moreover, N content would be of greater practical interest, because it is more directly related to photosynthetic rates. Fig. 8 shows a significantly positive correlation between the REIP and both nitrogen concentration and content with only a slight difference in the fraction of variance

explained (R^2= 0.90 and 0.88, respectively). We see the same strong relationship in the case of SPAD readings. In both cases, the observed correlation seems to be the result of the strong association between chlorophyll and nitrogen contents. So, we could say also that the reflectance indices that can predict well the chlorophyll content could also predict well the nitrogen content, although this relationship could be species- as well as site-specific.

5.4.9. Assessing the generality of observed relationships

In order to assess the generality of some of the observed relationships, a thorough literature search was carried out using one of the bibliographic search engines available at the University of Bologna (ISI Web of Knowledge by Thomson Reuters). Published references on the application of the SPAD instrument or the REIP spectroscopic technique for the assessment of leaf chlorophyll content or N concentration were screened and selected if referring to forest or otherwise tree species, and if containing quantitative information in tabular or graphic form.

The results are presented in Fig. 9 for chlorophyll contents and in Fig. 10 for N concentrations.

A rather consistent relationship between SPAD and leaf chlorophyll content can be derived from the literature (Fig. 9A), which is broadly consistent with what observed in the present study on poplar trees. The relationship was often found to be slightly curvilinear, although it was commonly approximated by a linear regression; the fraction of overall variability explained varied quite widely between species. Despite the general consistency, the difference even between species from the same genus is apparent. The application of a standard mean relationship would often lead to very substantial errors, suggesting the need for species- (and perhaps site-) specific calibrations, as already suggested by (Pinkard et al. 2006). This has been suggested to be partly related to the confounding effects of leaf thickness and water content, as the reference signal of the SPAD meter, at 960 nm, is located close to a region of water absorption. However, it should be noted that the species-specific nature of the relationship

appears to apply also in the case of the REIP index (Fig. 9B), which should not be affected by leaf water content, although the limited number of studies on tree species should be noted.

Also for the assessment of leaf N concentration, results from the SPAD instruments are generally consistent, but with a rather large variability between species (Fig. 10), suggesting the need for a careful calibration of the relationship for the conditions of interest.

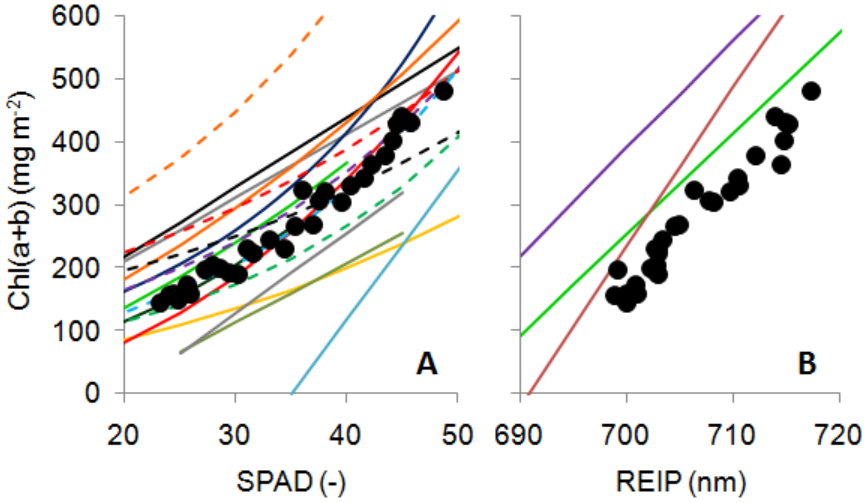

Fig. 9 Estimation of leaf chlorophyll content from **A** SPAD readings or **B** the red-edge index position (REIP) derived from spectroscopic measurements. Results for *P. euroamericana* (black dots) are compared with literature data for *P. deltoidex x nigra* and *P. nigra x maximowiczii* (grey and green; (Lombard et al. 2010), *Betula* spp. (dark green; McNamara and Pellett 2001), *B. papyrifera* (pale green; (Richardson et al. 2002), *B. pendula* (black; (Schaper and Chacko 1991), *Eucalyptus nitens* and *E. globulus* (dark blue and red; (Pinkard et al. 2006), *Malus domestica* (pale blue; (Campbell et al. 1990), *Mangifera indica* (black; (Schaper and Chacko 1991), *Coffea arabica* (yellow; (Netto et al. 2010), *Acer saccharum* (purple; (Vogelmann et al. 1993), *Liquidambar styraciflua* (brown; (Sims and Gamon 2002)

.

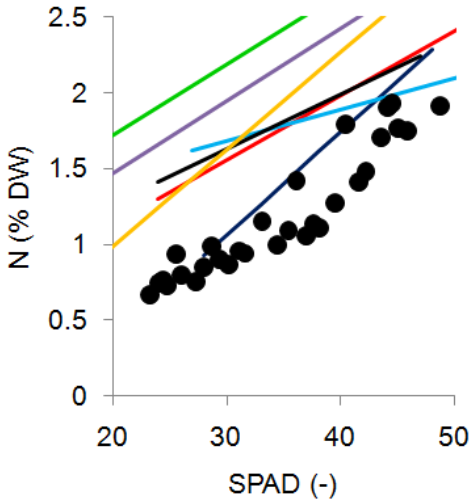

Fig. 10 Estimation of leaf N concentration from SPAD readings. Results for *P. euroamericana* (black dots) are compared with literature data for *Salix* (orange; (Weih and Ronnberg-Wastljung 2007), *Populus deltoides* (blue; (Loh et al. 2002), *P. tremuloides* (red; (Bonneville and Fyles 2006)and *P. heterophylla* (green), *Fraxinus pennsylvanica* (purple), *Platanus occidentalis* (black) and *Liquidambar styraciflua* (pale blue) from (Chang and Robison 2003).

5.5. *Conclusion*

The chlorophyll meter (SPAD-502) and the LICOR Li-1800 Spectroradiometer showed to be practical and convenient tools for monitoring plant growth development, and assess its nutritional status indirectly based on the reflectance properties of plant leaves.

We found that SPAD readings and the red-edge index position are good predictors of total chlorophyll content and carotenoids, proposing both as suitable instruments for the prediction of the nutritional status of vegetation. On the other hand, it was found not to be possible to extrapolate the relationship from fertilized treatments to unfertilized ones or vice versa. Together with the results from a re-analysis of published values for a number of tree species, this highlights the need for a careful on-site calibration of each relationship over the widest possible range of leaf nutrients.

The strong relationship found between PRI and the carotenoids/chlorophyll ratio in response to fertilization reinforce what has been found before about PRI as an efficient remote sensing reflectance index estimating not only short-term changes in photochemical efficiency of plants, but also long-term changes in the biochemistry of the photosynthetic apparatus (Garbulsky et al. 2011).

These results are of considerable importance to the practice of management of poplar plantations, in order not to waste fertilizer, if not needed, and not to risk a loss of productivity on the contrary if there was a need for fertilization.

Reference list

Barigah, T.S., Saugier, B., Mousseau, M., Guittet, J., & Ceulemans, R. (1994). Photosynthesis, Leaf-Area and Productivity of 5 Poplar Clones During Their Establishment Year. *Annales des Sciences Forestieres, 51*, 613-625

Bausch, W.C., & Duke, H.R. (1996). Remote sensing of plant nitrogen status in corn. *Transactions of the Asae, 39*, 1869-1875

Blackburn, G.A. (1998). Spectral indices for estimating photosynthetic pigment concentrations: a test using senescent tree leaves. *International Journal of Remote Sensing, 19*, 657-675

Bonneville, M.C., & Fyles, J. (2006). Assessing variations in SPAD-502 chlorophyll meter measurements and their relationships with nutrient content of trembling aspen foliage. *Communications in Soil Science and Plant Analysis, 37*, 525-539

Boochs, F., Kupfer, G., Dockter, K., & Kuhbauch, W. (1990). Shape of the Red Edge As Vitality Indicator for Plants. *International Journal of Remote Sensing, 11*, 1741-1753

Buckland, S.M., Price, A.H., & Hendry, G.A.F. (1991). The Role of Ascorbate in Drought-Treated Cochlearia-Atlantica Pobed and Armeria-Maritima (Mill) Willd. *New Phytologist, 119*, 155-160

Buschmann, C., & Nagel, E. (1993). Invivo Spectroscopy and Internal Optics of Leaves As Basis for Remote-Sensing of Vegetation. *International Journal of Remote Sensing, 14*, 711-722

Campbell, R.J., Mobley, K.N., Marini, R.P., & Pfeiffer, D.G. (1990). GROWING CONDITIONS ALTER THE RELATIONSHIP BETWEEN SPAD-501 VALUES AND APPLE LEAF CHLOROPHYLL. *Hortscience, 25*, 330-331

Carter, G.A., & Knapp, A.K. (2001). Leaf optical properties in higher plants: Linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany, 88*, 677-684

Chang, S.X., & Robison, D.J. (2003). Nondestructive and rapid estimation of hardwood foliar nitrogen status using the SPAD-502 chlorophyll meter. *Forest Ecology and Management, 181*, 331-338

Chappelle, E.W., Kim, M.S., & McMurtrey, J.E. (1992). Ratio Analysis of Reflectance Spectra (Rars) - An Algorithm for the Remote Estimation of the Concentrations of Chlorophyll-A, Chlorophyll-B, and Carotenoids in Soybean Leaves. *Remote Sensing of Environment, 39*, 239-247

Cho, M.A., & Skidmore, A.K. (2006). A new technique for extracting the red edge position from hyperspectral data: The linear extrapolation method. *Remote Sensing of Environment, 101*, 181-193

Clevers, J.G.P.W., De Jong, S.M., Epema, G.F., Van der Meer, F.D., Bakker, W.H., Skidmore, A.K., & Scholte, K.H. (2002). Derivation of the red edge index using the MERIS standard band setting. *International Journal of Remote Sensing, 23*, 3169-3184

Collins, W. (1978). Remote sensing of crop type and maturity. *Photogrammetric Engineering and Remote Sensing, 44*, 43-55.

Curran, P.J., Dungan, J.L., & Gholz, H.L. (1990). Exploring the Relationship Between Reflectance Red Edge and Chlorophyll Content in Slash Pine. *Tree Physiology, 7*, 33-48

Curran, P.J., Dungan, J.L., Macler, B.A., & Plummer, S.E. (1991). The Effect of A Red Leaf Pigment on the Relationship Between Red Edge and Chlorophyll Concentration. *Remote Sensing of Environment, 35*, 69-76

Curran, P.J., Windham, W.R., & Gholz, H.L. (1995). Exploring the Relationship Between Reflectance Red Edge and Chlorophyll Concentration in Slash Pine Leaves. *Tree Physiology, 15*, 203-206

Dawson, T.P., & Curran, P.J. (1998). A new technique for interpolating the reflectance red edge position. *International Journal of Remote Sensing, 19*, 2133-2139

Demmigadams, B. (1990). Carotenoids and Photoprotection in Plants - A Role for the Xanthophyll Zeaxanthin. *Biochimica et Biophysica Acta, 1020*, 1-24

Demmigadams, B., & Adams, W.W. (1996). The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends in Plant Science, 1*, 21-26

Everitt, J.H., Richardson, A.J., & Gausman, H.W. (1985). Leaf Reflectance-Nitrogen-Chlorophyll Relations in Buffelgrass. *Photogrammetric Engineering and Remote Sensing, 51*, 463-466

Filella, I., Amaro, T., Araus, J.L., & Pe¤uelas, J. (1996). Relationship between photosynthetic radiation-use efficiency of barley canopies and the photochemical reflectance index (PRI). *Physiologia Plantarum, 96*, 211-216

Filella, I., & Penuelas, J. (1994). The Red Edge Position and Shape As Indicators of Plant Chlorophyll Content, Biomass and Hydric Status. *International Journal of Remote Sensing, 15*, 1459-1470

Filella, I., Porcar-Castell, A., Munne-Bosch, S., Back, J., Garbulsky, M.F., & Penuelas, J. (2009). PRI assessment of long-term changes in carotenoids/chlorophyll ratio and short-term changes in de-epoxidation state of the xanthophyll cycle. *International Journal of Remote Sensing, 30*, 4443-4455

Filella, I., Serrano, L., Serra, J., & Penuelas, J. (1995). Evaluating Wheat Nitrogen Status with Canopy Reflectance Indexes and Discriminant-Analysis. *Crop Science, 35*, 1400-1405

Gamon, J.A., Field, C.B., Fredeen, A.L., & Thayer, S. (2001). Assessing photosynthetic downregulation in sunflower stands with an optically-based model. *Photosynthesis Research, 67*, 113-125

Gamon, J.A., Penuelas, J., & Field, C.B. (1992). A Narrow-Waveband Spectral Index That Tracks Diurnal Changes in Photosynthetic Efficiency. *Remote Sensing of Environment, 41*, 35-44

Gamon, J.A., & Qiu, H.L. (1999). Ecological applications of remote sensing at multiple scales. *Handbook of Functional Plant Ecology*, 805-846

Gamon, J.A., Serrano, L., & Surfus, J.S. (1997). The photochemical reflectance index: an optical indicator of photosynthetic radiation use efficiency across species, functional types, and nutrient levels. *Oecologia, 112*, 492-501

Gamon, J.A., & Surfus, J.S. (1999). Assessing leaf pigment content and activity with a reflectometer. *New Phytologist, 143*, 105-117

Garbulsky, M.F., Penuelas, J., Gamon, J., Inoue, Y., & Filella, I. (2011). The photochemical reflectance index (PRI) and the remote sensing of leaf, canopy and ecosystem radiation use efficiencies A review and meta-analysis. *Remote Sensing of Environment, 115*, 281-297

Garbulsky, M.F., Penuelas, J., Papale, D., & Filella, I. (2008). Remote estimation of carbon dioxide uptake by a Mediterranean forest. *Global Change Biology, 14*, 2860-2867

Garrity, S.R., Eitel, J.U.H., & Vierling, L.A. (2011). Disentangling the relationships between plant pigments and the photochemical reflectance index reveals a new approach for remote estimation of carotenoid content. *Remote Sensing of Environment, 115*, 628-635

Gitelson, A., & Merzlyak, M.N. (1994a). Quantitative Estimation of Chlorophyll-A Using Reflectance Spectra - Experiments with Autumn Chestnut and Maple Leaves. *Journal of Photochemistry and Photobiology B-Biology, 22*, 247-252

Gitelson, A., & Merzlyak, M.N. (1994b). Spectral Reflectance Changes Associated with Autumn Senescence of Aesculus-Hippocastanum l and Acer-Platanoides l Leaves - Spectral Features and Relation to Chlorophyll Estimation. *Journal of Plant Physiology, 143*, 286-292

Haboudane, D., Miller, J.R., Pattey, E., Zarco-Tejada, P.J., & Strachan, I.B. (2004). Hyperspectral vegetation indices and novel algorithms for predicting green LAI of crop canopies: Modeling and validation in the context of precision agriculture. *Remote Sensing of Environment, 90*, 337-352

Hansen, P.M., & Schjoerring, J.K. (2003). Reflectance measurement of canopy biomass and nitrogen status in wheat crops using normalized difference vegetation indices and partial least squares regression. *Remote Sensing of Environment, 86*, 542-553

Horler, D.N.H., Barber, J., & Barringer, A.R. (1980). Effects of heavy metals on the absorbance and reflectance spectra of plants. *International Journal of Remote Sensing, 1*, 121-136

Horler, D. N. H., Dockray, M., & Barber, J. (1983). The red edge of plant leaf reflectance. *International Journal of Remote Sensing, 4(2),* 273-288.

Inada, K. (1985). Spectral Ratio of Reflectance for Estimating Chlorophyll Content of Leaf. *Japanese Journal of Crop Science, 54*, 261-265

Jaynes, D.B., Colvin, T.S., Karlen, D.L., Cambardella, C.A., & Meek, D.W. (2001). Nitrate loss in subsurface drainage as affected by nitrogen fertilizer rate. *Journal of Environmental Quality, 30*, 1305-1314

Lamb, D.W., Steyn-Ross, M., Schaare, P., Hanna, M.M., Silvester, W., & Steyn-Ross, A. (2002). Estimating leaf nitrogen concentration in ryegrass (Lolium spp.) pasture using the chlorophyll

red-edge: theoretical modelling and experimental observations. *International Journal of Remote Sensing, 23*, 3619-3648
Lichtenthaler, H.K., & Wellburn, A.R. (1983). Determinations of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochem. Soc. Trans, 11*, 591-592

Loh, F.C.W., Grabosky, J.C., & Bassuk, N.L. (2002). Using the SPAD 502 meter to assess chlorophyll and nitrogen content of benjamin fig and cottonwood leaves. *Horttechnology, 12*, 682-686

Lombard, K., O'Neill, M., Mexal, J., Ulery, A., Onken, B., Bettmann, G., & Heyduck, R. (2010). Can soil plant analysis development values predict chlorophyll and total Fe in hybrid poplar? *Agroforestry Systems, 78*, 1-11

Mariotti, M., Ercoli, L., & Masoni, A. (1996). Spectral properties of iron-deficient corn and sunflower leaves. *Remote Sensing of Environment, 58*, 282-288

Marquard, R.D., & Tipton, J.L. (1987). Relationship Between Extractable Chlorophyll and An Insitu Method to Estimate Leaf Greenness. *Hortscience, 22*, 1327-1327

McMurtrey, J.E., Chappelle, E.W., Kim, M.S., Meisinger, J.J., & Corp, L.A. (1994). Distinguishing Nitrogen-Fertilization Levels In-Field Corn (Zea-Mays L) with Actively Induced Fluorescence and Passive Reflectance Measurements. *Remote Sensing of Environment, 47*, 36-44

McNamara, S., & Pellett, H. (2001). Effect of high pH on foliar chlorosis and growth of five Betula species. *Journal of Environmental Horticulture*, *19 (4)*, 175-179

Merzlyak, M.N., Gitelson, A.A., Chivkunova, O.B., & Rakitin, V.Y. (1999). Non-destructive optical detection of pigment changes during leaf senescence and fruit ripening. *Physiologia Plantarum, 106*, 135-141

Moran, J.A., Mitchell, A.K., Goodmanson, G., & Stockburger, K.A. (2000). Differentiation among effects of nitrogen fertilization treatments on conifer seedlings by foliar reflectance: a comparison of methods. *Tree Physiology, 20*, 1113-1120

Netto, A.T., Campostrini, E., de Oliveira, J.G., & Bressan-Smith, R.E. (2005). Photosynthetic pigments, nitrogen, chlorophyll a fluorescence and SPAD-502 readings in coffee leaves. *Scientia Horticulturae, 104*, 199-209

Netto, A.T., Campostrini, E., Goncalves de Oliveira, J., & Bressan-Smith, R.E. (2010). Photosynthetic pigments, nitrogen, chlorophyll a fluorescence and SPAD-502 readings in coffee leaves. *Scientia Horticulturae, 104*, 199-209

Nichol, C.J., Huemmrich, K.F., Black, T.A., Jarvis, P.G., Walthall, C.L., Grace, J., & Hall, F.G. (2000). Remote sensing of photosynthetic-light-use efficiency of boreal forest. *Agricultural and Forest Meteorology, 101*, 131-142

Ort, D.R. (2001). When there is too much light. *Plant Physiology, 125*, 29-32
Peng, S.B., Laza, M.R.C., Garcia, F.V., & Cassman, K.G. (1995). Chlorophyll Meter Estimates Leaf Area-Based Nitrogen Concentration of Rice. *Communications in Soil Science and Plant Analysis, 26*, 927-935

Penuelas, J., Baret, F., & Filella, I. (1995). Semiempirical Indexes to Assess Carotenoids Chlorophyll-A Ratio from Leaf Spectral Reflectance. *Photosynthetica, 31*, 221-230

Penuelas, J., & Filella, I. (1998). Visible and near-infrared reflectance techniques for diagnosing plant physiological status. *Trends in Plant Science, 3*, 151-156

Penuelas, J., Gamon, J.A., Fredeen, A.L., Merino, J., & Field, C.B. (1994). Reflectance Indexes Associated with Physiological-Changes in Nitrogen-Limited and Water-Limited Sunflower Leaves. *Remote Sensing of Environment, 48*, 135-146

Penuelas, J., & Inoue, Y. (2000). Reflectance assessment of canopy CO2 uptake. *International Journal of Remote Sensing, 21*, 3353-3356

Penuelas, J., Llusia, J., Pinol, J., & Filella, I. (1997). Photochemical reflectance index and leaf photosynthetic radiation-use-efficiency assessment in Mediterranean trees. *International Journal of Remote Sensing, 18*, 2863-2868

Peterson, D.L., Aber, J.D., Matson, P.A., Card, D.H., Swanberg, N., Wessman, C., & Spanner, M. (1988). Remote-Sensing of Forest Canopy and Leaf Biochemical Contents. *Remote Sensing of Environment, 24*, 85-&

Pinkard, E.A., Patel, V., & Mohammed, C. (2006). Chlorophyll and nitrogen determination for plantation-grown Eucalyptus nitens and E-globulus using a non-destructive meter. *Forest Ecology and Management, 223*, 211-217

Richardson, A.D., Duigan, S.P., & Berlyn, G.P. (2002). An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist, 153*, 185-194

Schaper, H., & Chacko, E.K. (1991). Relation Between Extractable Chlorophyll and Portable Chlorophyll Meter Readings in Leaves of 8 Tropical and Subtropical Fruit-Tree Species. *Journal of Plant Physiology, 138*, 674-677

Seel, W.E., Hendry, G.A.F., & Lee, J.A. (1992). The Combined Effects of Desiccation and Irradiance on Mosses from Xeric and Hydric Habitats. *Journal of Experimental Botany, 43*, 1023-1030

Sims, D.A., & Gamon, J.A. (2002). Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment, 81*, 337-354

Smeal, D., & Zhang, H. (1994). Chlorophyll Meter Evaluation for Nitrogen Management in Corn. *Communications in Soil Science and Plant Analysis, 25*, 1495-1503

Smith, K.L., Steven, M.D., & Colls, J.J. (2004). Use of hyperspectral derivative ratios in the red-edge region to identify plant stress responses to gas leaks. *Remote Sensing of Environment, 92*, 207-217

Stylinski, C.D., Gamon, J.A., & Oechel, W.C. (2002). Seasonal patterns of reflectance indices, carotenoid pigments and photosynthesis of evergreen chaparral species. *Oecologia, 131*, 366-374

Sullivan, D.G., Shaw, J.N., Mask, P.L., Rickman, D., Luvall, J., & Wersinger, J.M. (2004). Evaluating corn nitrogen variability via remote-sensed data. *Communications in Soil Science and Plant Analysis, 35*, 2465-2483

Trotter, G.M., Whitehead, D., & Pinkney, E.J. (2002). The photochemical reflectance index as a measure of photosynthetic light use efficiency for plants with varying foliar nitrogen contents. *International Journal of Remote Sensing, 23*, 1207-1212

Vogelmann, J.E., Rock, B.N., & Moss, D.M. (1993). RED EDGE SPECTRAL MEASUREMENTS FROM SUGAR MAPLE LEAVES. *International Journal of Remote Sensing, 14*, 1563-1575

Weih, M., & Ronnberg-Wastljung, A.C. (2007). Shoot biomass growth is related to the vertical leaf nitrogen gradient in Salix canopies. *Tree Physiology, 27*, 1551-1559

Woolley, J.T. (1971). Reflectance and Transmittance of Light by Leaves. *Plant Physiology, 47*, 656-&

Yoder, B.J., & Pettigrewcrosby, R.E. (1995). Predicting Nitrogen and Chlorophyll Content and Concentrations from Reflectance Spectra (400-2500 Nm) at Leaf and Canopy Scales. *Remote Sensing of Environment, 53*, 199-211

Chapter 6

6.1. General discussion

More attention is being given to non-invasive remote sensing techniques for monitoring plant photosynthetic and stress status. These techniques depend on the detection of the energy partitioning in leaves and the reflectance properties related.

Some of these missions are accomplished by the remotely sensing of chlorophyll fluorescence influenced by changes in reflectance in the red-edge spectral region, affected by energy dissipation as a result of excess light (Horton et al. 1994). As chlorophyll fluorescence is emitted primarily from chlorophyll-*a* of the antenna system of PSII, so any physiological process that influences the function of PSII will have an effect of chlorophyll fluorescence (Horton et al. 1994). Our results confirm these studies when we found a strong and consistent correlation between PSII electron transport and fluorescence radiance, besides that fluorescence yield (Φ_f) was changing with changes of photochemical yield (Φ_{PSII}), and following variations of environmental conditions (irradiance intensity or CO2 concentrations). These findings are consistent with previous studies that correlate F_t with diurnal variation in stomatal conductance (g) and CO2 assimilation (A) as a result of variation in irradiance and water stress conditions (Flexas et al. 2000; Flexas et al. 2002; Flexas et al. 1999). But we should take in consideration that leaves absorbance (α) is not constant, because of the variation in chlorophyll and nitrogen content, and because just chlorophyll-a is the responsible for the dominant fluorescence emission, so we have to correct the fluorescence data to absorbance. These results were found similarly in two contrasting species (*Arbutus unedo*, a schlerophyllous Mediterranean species, and *Populus euroamericana*, a broad leaf deciduous tree).

So, we can say that ambient fluorescence could provide a useful tool for testing photosynthetic processes from a distance, even if fluorescence signal needs

moor efforts for accurate interpretation. That will open the way for long-term monitoring of plant stress conditions using passive remote sensing strategies.

Other methods used for the remote sensing of the photosynthetic processes depend on the detection of changes in photosynthetic pigments. Several studies in the remote sensing have developed a strong link between spectral reflectance measurements and plant photosynthetic pigments concentrations (Sims and Gamon 2002). Many vegetation reflectance indices was created to assess potential levels of plants' photosynthesis and net primary productivity (Gamon et al 1995) by monitoring changes in plant photosynthetic status brought on by environmental conditions. One of these indices is the photochemical reflectance index (PRI). It remotely detects the variation in xanthophylls cycle (Gamon et al. 1990; Gamon et al. 1995; Gamon et al. 1992; Gamon et al. 1997). This index estimates variations of xanthophylls cycle pigments as a result of changes in photosynthetic light use efficiency (Gamon et al. 1992; Gamon et al. 1997; Penuelas et al. 1995b; Penuelas et al. 1997). It uses spectral bands located in the absorption region of both chlorophyll and carotenoid pigments. Consequently, it has been shown to be sensitive to carotenoid/chlorophyll ratio across a number of species (Sims and Gamon 2002). Our results come to add a new evidence for these findings as we found a strong relationship between PRI and the carotinoids/chlorophyll ratio, which is even better than what was found in the literature with an $r^2 = 0.73$. However, PRI can not be used as a predictor of photochemical yield over long period. Over the course of one day, the chlorophyll content, or the nitrogen content or the ratio between carotenoides and chlorophyll will remain constant, and therefore we will have a good relationship between PRI and photochemical yield. But over the long term, we will have changes in nitrogen or chlorophyll concentration or other photosynthetic pigments, and this will have an effect on PRI independent of the effect on photochemical yield. And it is not possible to discriminate between the two components.

These new techniques used for remote sensing of photosynthetic processes have been shown great efficiency detecting plant growth development and nutritional status. Different methods are being used in this domain, among them the estimation of chlorophyll content using SPAD-502, and the reflectance with the method of the red-edge index position (REIP). As expected, we found a

strong correlation between the red-edge index position and the total chlorophyll content. The same linear relationship was found also between SPAD readings and the total chlorophyll content. And as measurements of nitrogen content can be achieved indirectly by quantifying chlorophyll content (Filella et al. 1995; Moran et al. 2000; Richardson et al. 2002) because of the close correlation between leaf chlorophyll concentration and nitrogen availability (Boochs et al. 1990; Everitt et al. 1985; Yoder and Pettigrewcrosby 1995), we can say that these methods can predict well the nutritional status of our plants. That was confirmed by the significantly positive correlation between the REIP and both of the nitrogen concentration and content we found, and the same strong relationship in the case of SPAD readings. So, these techniques can be used for reliable estimation of photosynthetic pigment content and total nitrogen. They can also assess the developments of photochemical process in our plants' leaves in the field, and that will help in the management of tree cultivation systems; and therefore estimating the nutrient requirements of our plants for optimal growth.

6.2. Conclusions

From the functional relationship between photosynthesis and ambient chlorophyll fluorescence measured under controlled conditions (variable CO_2, PAR) we can say that ambient fluorescence could provide a useful tool for testing photosynthetic processes from a distance. Problems remain however in its interpretation, despite new models being proposed. The experimental test demonstrates a consistent pattern of co-variation between fluorescence and photochemical yield. However, the relationship differs depending on whether photosynthesis is limited by light (negative association) or CO_2 (positive association). It is rather similar the response in two different species: one is a broad leaf sclerofillous tree from the Mediterranean (*Arbutus unedo*), and the other is broad leaf deciduous tree *(Populus euroamericana*). There are some differences between the two species, but the general pattern is the same with variable PAR and variable CO_2.

The new understanding of ambient fluorescence opens novel perspectives for the airborne and satellite remote sensing of photosynthetic processes

Non-destructive monitoring methods, a direct application of RS studies, are increasingly attractive for the determination of stress conditions or nutrient deficiencies not only in research but also in agronomy, horticulture and urban forestry (*proximal remote sensing*).

Remote sensing techniques showed to be practical and convenient tools for monitoring plant growth development, and assess its nutritional status indirectly depending on the reflectance properties of plant leaves. So, they are of considerable importance to the practice of plants cultivation management and optimize fertilization for optimal growth. So, it is a much applied research.

Reference list

Boochs, F., Kupfer, G., Dockter, K., & Kuhbauch, W. (1990). Shape of the Red Edge As Vitality Indicator for Plants. *International Journal of Remote Sensing, 11*, 1741-1753

Everitt, J.H., Richardson, A.J., & Gausman, H.W. (1985). Leaf Reflectance-Nitrogen-Chlorophyll Relations in Buffelgrass. *Photogrammetric Engineering and Remote Sensing, 51*, 463-466

Filella, I., Porcar-Castell, A., Munne-Bosch, S., Back, J., Garbulsky, M.F., & Penuelas, J. (2009). PRI assessment of long-term changes in carotenoids/chlorophyll ratio and short-term changes in de-epoxidation state of the xanthophyll cycle. *International Journal of Remote Sensing, 30*, 4443-4455

Filella, I., Serrano, L., Serra, J., & Penuelas, J. (1995). Evaluating Wheat Nitrogen Status with Canopy Reflectance Indexes and Discriminant-Analysis. *Crop Science, 35*, 1400-1405

Flexas, J., Briantais, J.M., Cerovic, Z., Medrano, H., & Moya, I. (2000). Steady-state and maximum chlorophyll fluorescence responses to water stress in grapevine leaves: A new remote sensing system. *Remote Sensing of Environment, 73*, 283-297

Flexas, J., Escalona, J.M., Evain, S., Gulias, J., Moya, I., Osmond, C.B., & Medrano, H. (2002). Steady-state chlorophyll fluorescence (Fs) measurements as a tool to follow variations of net CO2 assimilation and stomatal conductance during water-stress in C-3 plants. *Physiologia Plantarum, 114*, 231-240

Flexas, J., Escalona, J.M., & Medrano, H. (1999). Water stress induces different levels of photosynthesis and electron transport rate regulation in grapevines. *Plant Cell and Environment, 22*, 39-48

Gamon, J.A., Field, C.B., Bilger, W., Bjorkman, O., Fredeen, A.L., & Penuelas, J. (1990). Remote-Sensing of the Xanthophyll Cycle and Chlorophyll Fluorescence in Sunflower Leaves and Canopies. *Oecologia, 85*, 1-7

Gamon, J.A., Field, C.B., Goulden, M.L., Griffin, K.L., Hartley, A.E., Joel, G., Penuelas, J., & Valentini, R. (1995). Relationships between ndvi, canopy structure, and photosynthesis in 3 californian vegetation types. *Ecological Applications, 5*, 28-41

Gamon, J.A., Penuelas, J., & Field, C.B. (1992). A Narrow-Waveband Spectral Index That Tracks Diurnal Changes in Photosynthetic Efficiency. *Remote Sensing of Environment, 41*, 35-44

Gamon, J.A., Serrano, L., & Surfus, J.S. (1997). The photochemical reflectance index: an optical indicator of photosynthetic radiation use efficiency across species, functional types, and nutrient levels. *Oecologia, 112*, 492-501

Horton, P., Ruban, A.V., & Walters, R.G. (1994). Regulation of light-harvesting in green plants - indication by nonphotochemical quenching of chlorophyll fluorescence. *Plant Physiology, 106*, 415-420

Merzlyak, M.N., Gitelson, A.A., Chivkunova, O.B., & Rakitin, V.Y. (1999). Non-destructive optical detection of pigment changes during leaf senescence and fruit ripening. *Physiologia Plantarum, 106*, 135-141

Moran, J.A., Mitchell, A.K., Goodmanson, G., & Stockburger, K.A. (2000). Differentiation among effects of nitrogen fertilization treatments on conifer seedlings by foliar reflectance: a comparison of methods. *Tree Physiology, 20*, 1113-1120

Penuelas, J., Baret, F., & Filella, I. (1995a). Semiempirical Indexes to Assess Carotenoids Chlorophyll-A Ratio from Leaf Spectral Reflectance. *Photosynthetica, 31*, 221-230

Penuelas, J., & Filella, I. (1998). Visible and near-infrared reflectance techniques for diagnosing plant physiological status. *Trends in Plant Science, 3*, 151-156

Penuelas, J., Filella, I., & Gamon, J.A. (1995b). Assessment of Photosynthetic Radiation-Use Efficiency with Spectral Reflectance. *New Phytologist, 131*, 291-296

Penuelas, J., Llusia, J., Pinol, J., & Filella, I. (1997). Photochemical reflectance index and leaf photosynthetic radiation-use-efficiency assessment in Mediterranean trees. *International Journal of Remote Sensing, 18*, 2863-2868

Richardson, A.D., Duigan, S.P., & Berlyn, G.P. (2002). An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytologist, 153*, 185-194

Sims, D.A., & Gamon, J.A. (2002). Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment, 81*, 337-354

Stylinski, C.D. (2000). Effects of Resource Availability on Plant Reflectance and Physiology. In (p. 134): University of California, Davis

Yoder, B.J., & Pettigrewcrosby, R.E. (1995). Predicting Nitrogen and Chlorophyll Content and Concentrations from Reflectance Spectra (400-2500 Nm) at Leaf and Canopy Scales. *Remote Sensing of Environment, 53*, 199-211