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Application of a chlorophyll fluorescence sensor to detect chelate-induced metal stress in *Zea mays*

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Abstract

Metal stress was induced in maize (*Zea mays* L.) by the addition to the soil of a range of concentrations of either ethylene-diamine-tetra-acetate (EDTA) or citric acid (CA) as chelating agents. Measurements were taken using a recently-developed sensor capable of plant fluorescence detection at wavelengths of 762 and 688 nm. Atmospheric oxygen absorbs radiation at these wavelengths. As such, measured fluorescence can be attributed to the plants under observation. Red/far-red (690/760 nm, R/FR) chlorophyll (Chl) fluorescence ratios were measured before addition of the chelating agents and during the month following. Significant differences were seen in the fluorescence responses of those plants for which high concentrations [$\geq$ 30 mmol kg^{-1}(d.m. soil)] of EDTA were added to the pots compared to those for which CA or no chelating agent was added. The plants for which high concentrations of EDTA were added also exhibited higher tissue metal concentrations and demonstrated visible signs of stress. Before signs of visual stress became apparent, R/FR Chl fluorescence ratios for metal-stressed plants were significantly different to those observed for unstressed plants. These results support the use of plant fluorescence as a potential tool for early indication of phytotoxic metal stress.

Additional key words: biomass; cadmium; citric acid; EDTA; maize; phytoremediation; phytotoxicity; plant stress; R/FR ratio.

Introduction

Under normal physiological conditions the majority of photons absorbed by plants is used for photochemistry (*i.e*. photosynthesis) and only a small proportion is dissipated through other mechanisms (Lichtenthaler and Miehé 1997). In higher plants, radiation in the photosynthetically active range ($400<\lambda<700$ nm) is absorbed and converted into energy suitable for use by the photosynthetic reaction. This absorption takes place in a protein/pigment bed called the light-harvesting complex (Lichtenthaler *et al.* 1982, Rosenqvist and van Kooten 2003). Absorption of photons causes excitation of chlorophyll (Chl). In order for plants to use the photon energy, de-excitation is needed *via* the photochemical reactions of photosynthesis. In healthy leaves about 90 % of the absorbed radiant energy is funnelled into reaction centres (Genty and Harbinson 1996). Although the majority of excess energy is dissipated as heat, some of the energy not used for photochemistry is released as fluorescence (Lichtenthaler 1996). When plants are subjected to increased stress the rate of photosynthesis drops, and the fraction of absorbed energy released as fluorescence and heat increases.

Although Chl absorbs photons strongly in both the blue and red parts of the spectrum, its fluorescence is entirely in the red region. The wavelength for fluorescence is almost coincident with the red part of the absorption spectrum (Nobel 1999). The fluorescence peak of extracted Chl is at 666 nm. *In vivo*, the fluorescence emission and absorption spectra are "red-shifted" as a result of being bound to proteins (Harbinson and Rosenqvist 2003). In leaves and other plant tissues two main emission bands occur at 685 and 735-740 nm (Lichtenthaler and Buschmann 1987, Franck *et al*. 2002). The fluorescence band at 685 nm arises from de-excitation of Chl molecules that have absorbed photons at the peak absorption band. *In vitro* measurements of the emission spectra show that the 666 nm peak is much higher than the far-red peak. However, *in vivo* re-absorption of short wavelength fluorescence (less than 700 nm) reduces the intensity of the red fluorescence emission to a value that is not much higher than the far-red emission (Lichtenthaler and Buschmann 1987, Harbinson and Rosenqvist 2003).

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Gitelson *et al.* (1998) have shown that 95 % of the changes in the R/FR ratio can be accounted for by re-absorption of the emitted photosystem 2 (PS2) fluorescence. Hence the contribution of photosystem 1 (PS1) Chl fluorescence to total fluorescence emission at room temperature can only be around 5 %.

Historically, study of Chl fluorescence has largely been devoted to understanding the mechanisms and controls of photosynthesis. More recently, the development of reliable fluorescence meters featuring pre-programmed protocols for taking measurements has changed the focus of research activity. A number of sensors have been developed that use fluorescence induced by artificial light sources to monitor plant photosynthesis. The Plant Health Monitor (PHM, *Aerodyne*, Massachusetts, USA) is one such recently-developed fluorescence sensor. The design and construction of a passive version of the PHM are described in detail in Kebabian *et al.* (1999). Previously, fluorescence measurement had been a laboratory technique with limited commercial uses, due to the need both for a dark environment and for the "dark adaptation" of the plant tissue prior to testing (Norikane *et al.* 2003). Under sunlight, Chl in plants fluoresces at wavelengths between 660 and 800 nm, a wavelength range which lies close to the peak of the solar spectrum. One of the key factors that have previously prevented the use of Chl fluorescence as a remote diagnostic tool is the inability to discriminate between scattered sunlight and radiation emitted by the plant. In the case of the PHM, the plant material is irradiated by a modulated blue LED source and the induced fluorescence measured using synchronous detection techniques. Hence measurements can be taken in sunlight. In practice, the ratio of fluorescence intensities at two wavelengths is usually determined, rather than the absolute intensities themselves.

The use of fluorescence meters for detecting plant stress is a major area of research, much of which has involved precision farming and the use of fluorescence data to improve the efficiency of agricultural inputs (Apostol *et al.* 2003). The utility of Chl fluorescence as an indicator of plant stress is that rapid and non-destructive measurements can be taken. A further use for the technology is in ecological monitoring. For example, work by the US Department of Energy describes the ultimate goal as field deployment of a remote sensing instrument suited for contamination detection, be it identification of problem areas, contaminant verification, monitoring of phytoremediators, or other issues through fluorescence signatures from plants (Capelle and Morgan 2003). Recently, fluorescence imaging systems have also been developed to monitor plant stress (Lichtenthaler and Babani 2000, Buschmann *et al.* 2000, Langsdorf *et al.* 2000).

Heavy metals occur widely as environmental pollutants resulting from industrial activity (Popovic *et al.* 2003), and the accumulation of heavy metals through the food chain can cause severe health problems; hence it is important that remediation technologies are developed. Phytoremediation technologies involve the extraction by plants of metals from the soil and concentrating them in above-ground parts such as leaves so that they can be harvested and removed from the affected sites. In order to enhance uptake of metals, chelating agents, which mobilise bonded metals and make them available to plants, may be added to the soil. At the University of Nottingham there is an ongoing research programme to evaluate the effectiveness of different chelating agents at a range of concentrations. For effective phytoremediation, enhancement of metal accumulation should not diminish the yield of the plant. Hence evaluation of plant health is required in order to assess the effectiveness of the remediation methodology (Barócsi *et al.* 2000).

The study reported here aimed to assess the effectiveness of the PHM as a tool in phytoremediation research. In this experiment, maize plants (*Zea mays*) were grown in soil contaminated with lead, cadmium, and zinc. Ethylene-diamine-tetra-acetate (EDTA) and citric acid (CA) were used as chelating agents and the *Aerodyne* PHM was used to monitor changes in leaf fluorescence levels.

Materials and methods

Plants: Maize seeds were sown in pots in glasshouse facilities at the University of Nottingham, UK. One week after germination, seedlings were transplanted into soil taken from the University's research site at Stoke Bardolph, Nottinghamshire, which has a history of contamination from sewage sludge. Prior to use the soil had been ground and sieved at 2 mm to improve homogeneity. Plants were watered regularly with deionised water to prevent drought stress. Chelating agents were added to the soil of four replicate pots 1 month after germination, at proportions of 5, 30, and 100 mmol kg^{-1}(dry mass soil).

Soil solution extraction: Samples of the soil solution were collected from pots subjected to the same treatments in order to give an indication of the levels of bioavailability of heavy metals. Soil solutions were extracted using rhizon samplers. After using a stainless steel rod to guide the tip of the sampler into the soil, an open syringe was connected to the rhizon sampler to draw up soil solution. Soil solutions were acidified with nitric acid and metal composition was analysed using flame atomic absorption spectrophotometry (F-AAS).

Measurement conditions: Chl fluorescence measurements were taken prior to the addition of the chelating agents and at intervals of 2–5 d during the following month. At the end of the measurement period, the plants were harvested. Fluorescence readings were taken inside

the greenhouse using the single leaf mode of the *Aerodyne* PHM, which required the attachment of a Fresnel lens to the front window of the monitor. In this mode, 20 % of the monitor's LEDs are used to irradiate the leaf. The leaf being analysed was supported 15 cm from the PHM and oriented perpendicular to the LED beam, which was focused on the upper surface of the leaf. Three readings were taken and averaged for each pot. Plants were harvested seven days after flowering, approximately one month after the addition of chelating agents to the soil.

Sensing of Chl fluorescence: The *Aerodyne* PHM operates by detecting radiation in the atmospheric A and B bands centred at 762 and 688 nm, respectively (Kebabian 1996). These bands correspond to wavelengths in the far-red and red Chl fluorescence bands. Radiation from fluorescing plants is passed through a cell containing oxygen at low pressure, which absorbs at 688 and 762 nm. Photons are emitted that are detected by a photomultiplier tube (a fluorescence-induced fluorescence response) (Freedman *et al.* 2002). The fluorescence signal is directly proportional to the intensity of the leaf fluorescence within the narrow oxygen absorption bands. Oxygen in the atmosphere also absorbs radiation at 688 and 762 nm. Therefore the effect of incident sunlight on readings is minimal.

The monitor reports three values: Chl fluorescence intensity at 690 nm (red – B band) and 760 nm (far-red – A band), and a ratio value R which contains a spectral responsivity correction. In addition, a function of the vegetation index (the fraction of the field of view filled by the plants), the distance between the plant and the monitor (the intensity falls off as the square of the distance) and the orientation of the leaf being viewed are recorded. Care was taken to minimise variation of these factors.

The ratio of red : far-red (R/FR) fluorescence was calculated using the recorded values for A (far-red) and B (red). The ratio has been reported as a measure of plant stress in a number of cases (Lichtenthaler and Miehé 1997, Barócsi *et al.* 2000, Langsdorf *et al.* 2000, Schuerger *et al.* 2003). The significance of the ratio has been attributed to two factors. First, Chl re-absorbs Chl fluorescence primarily at 690 nm (Lichtenthaler 1987, Gitelson *et al.* 1998). As such its intensity is related to Chl concentration. Second, the FR emission is less responsive to changes in the state of electron transport mechanisms than is the R emission (Freedman *et al*. 2002).

Plant analysis: Following flowering, plants were harvested 1 cm above the soil surface. Biomass was determined before the plant material was ground. Sub-samples (1 g) were digested in 70 % nitric acid (HNO_3), and analysed using F-AAS.

Results

The time courses of the fluorescence measurements are shown in Fig. 1. Where CA was used as a chelating agent there was little difference between the fluorescence ratios of the different treatments (Fig. 1*B*). However, there were differences observed in fluorescence when EDTA was used (Fig. 1*A*). Five days after the addition of chelating agents, fluorescence readings indicated that the R/FR ratio could be used to separate the 100 mmol EDTA treatment from all other treatments, even though at this stage there were no visual differences between the treatments. By seven days, there were visible signs of damage on the leaves of plants for which 100 mmol EDTA had been added to the soil. At this stage the R/FR ratio had increased. Eleven days after the addition of EDTA to the soil the plants in the 100 mmol treatment were taking up water at a far slower rate than the other treatment groups and the leaves were showing signs of cell death. Those leaves showing advanced stages of stress also showed reductions in the levels of measured fluorescence. Fig. 2 shows the R/FR ratios five and eleven days, respectively, after the addition of chelating agents.

Analysis of variance (ANOVA) on readings from day 5 indicated that plant fluorescence ratios could not be separated by treatment ($p>0.05$). However, the least significant difference of the means demonstrates that the R/FR ratio of the 100 mmol EDTA group is significantly different from all other treatments at the 5 % confidence limit.

Day 11 ANOVA readings suggest that differences between other treatments and the 100 mmol EDTA group are significant ($p\leq0.05$). At this stage the R/FR ratio was higher, and the R and FR fluorescence values were much lower than for all other treatments. At this point the plants were showing visible signs of stress.

The 30 mmol EDTA plants also showed clear visible signs of stress from 18 d after chelate application. There was a high variability of the individual fluorescence readings rather than any clear change in the R/FR ratio. Once plants began showing visible signs of stress, the measured A and B intensities were reduced. Fig. 3 shows the values measured 18 d after chelate application. ANOVA of recorded R and FR fluorescence values indicated that there were significant differences between treatments ($p<0.05$). The least significant difference of the means calculated at the 5 % level shows that FR fluorescence values for plants treated with 30 mmol EDTA were lower than those of all other treatments. The least significant difference of the means also indicates that values for R fluorescence were lower for the 30 mmol EDTA group than for the control.

Fig. 4*A* shows the effect of the chelating agents on the concentrations of cadmium in the soil solution. The soil

solution readings were taken six days after the addition of the chelating agents and are representative of the entire sampling period. The contents of cadmium present in the above-ground tissues of plants harvested 31 d after the addition of chelating agents are shown in Fig. 4*B*.

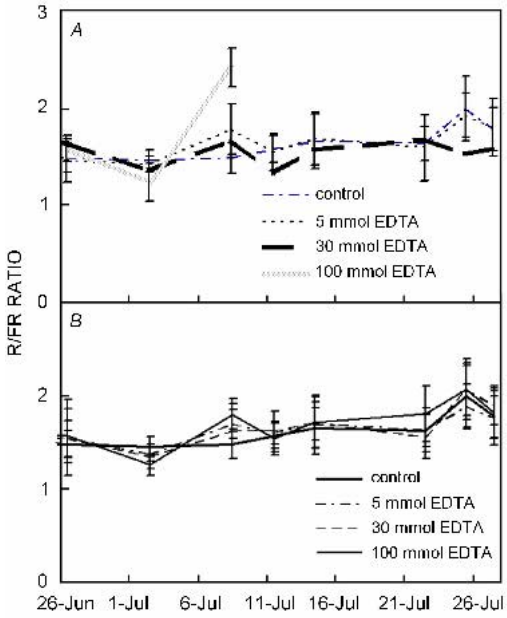

Fig. 1. Measured Red/Far Red (690/760 nm) Chl fluorescence ratios for untreated *Z. mays* plants and those treated with EDTA (*A*) or citric acid, CA (*B*). Chelating agents were added 27 June 2003. *Error bars* represent standard error.

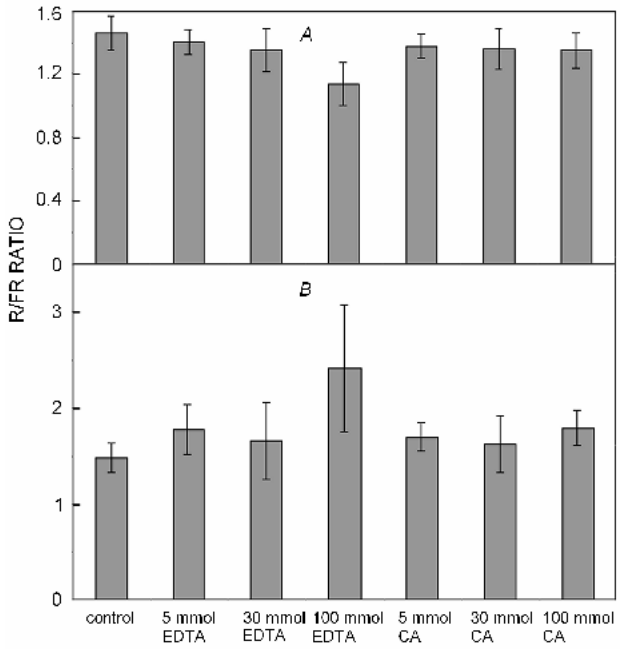

Fig. 2. R/FR Chl fluorescence ratios for *Z. mays,* grown under different chelate treatments, measured on 02 July 2003 (*A*) or 8 July 2003 (*B*). *Error bars* represent standard error. CA = citric acid.

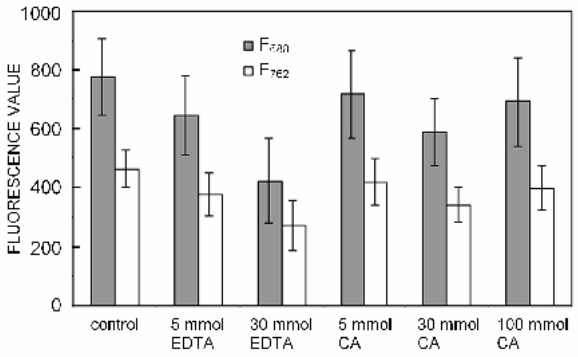

Fig. 3. Absolute red (F_{688}) and far red (F_{762}) values of Chl fluorescence measured by the *Aerodyne* PHM on 14 July 2003 for *Z. mays* grown under different chelate treatments. Note the significant reduction in fluorescence at 762 nm in the 30 mmol EDTA treatment. *Error bars* represent standard error. CA = citric acid.

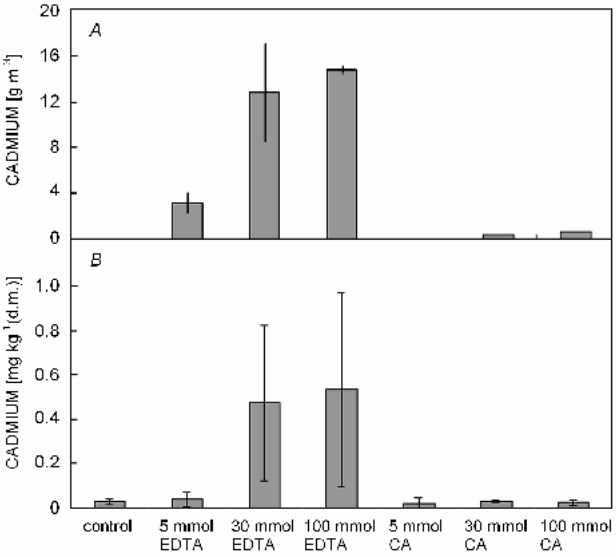

Fig. 4. Cadmium concentrations in soil solutions 6 d after treatment of the soil with EDTA and CA chelating agents (*A*) and cadmium contents per dry mass in *Z. mays* plants 31 d after addition of chelating agent to the soil (*B*). *Error bars* represent 1 standard deviation from the mean.

The increase in metal concentration in the soil solution as well as in shoots of maize as a result of EDTA addition is described by Cunningham and Ow (1996). Increases in the metal concentration alter root/shoot partitioning in crop plants. Although this can often result in rapid plant death, the crop often remains harvestable for metal removal. This appears to have been the case in this experiment with regard to those plants treated with 100 mmol EDTA. Biomass values for the plants following harvesting are shown in Fig. 5. Those plants that displayed visible levels of stress showed reduced biomass. It seems likely that this was due to damage to the photosynthetic apparatus. Of note was a significant increase (p<0.01) in the R/FR ratio after flowering, which may

have been due to decreasing Chl content during the beginnings of senescence. Increases in the R/FR ratio during senescence have been reported by Lichtenthaler (1987) and Subhash *et al*. (1999).

Discussion

A number of potential uses have been suggested for Chl fluorescence monitors. These include site-specific agriculture, irrigation on demand, hydroponic growing environments, and ecological monitoring. Various research groups have developed monitors that are capable of inducing fluorescence by use of artificial light sources. In this case the *Aerodyne* PHM was able to detect changes in the R/FR ratio before stresses became visually apparent.

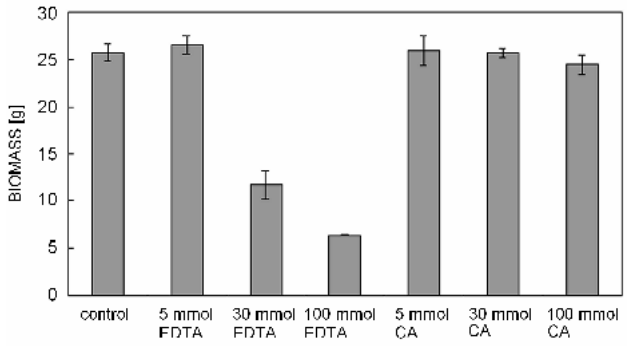

Fig. 5. Biomass measured for *Z. mays* plants, grown under different chelate treatments, 31 d after addition of chelating agent to the soil. *Error bars* represent 1 standard deviation of the mean. CA = citric acid.

Several authors have reported changes (generally an increase) to the R/FR ratio in plants under stress (Buschmann *et al.* 1996, Lichtenthaler and Miehé 1997, Chaerle and van der Straeten 2001). When chlorotic patches appeared on the leaves of the plants treated with 100 mmol EDTA, the R/FR ratio increased markedly. The main reason proposed for this effect is the reduction of re-absorption of red radiation as the contents of photosynthetic pigment are reduced (Lichtenthaler 1987, Lichtenthaler and Rinderle 1988, Schuerger *et al.* 2003). However, this fails to explain the initial fall in the R/FR values, following application of chelating agents. Barócsi *et al.* (2000) found that the fluorescence ratio F_{690}/F_{735} was not significantly influenced by the effect of heavy metals at an early stage. In this case the Chl fluorescence decay (peak fluorescence intensity/steady-state fluorescence) declined due to inhibition of photosynthetic activity. The *Aerodyne* PHM measures "steady-state" fluorescence and cannot be used to obtain time-resolved signals that extract kinetic information.

Heavy metals induce plant stress by inhibition of photosynthetic enzymes. Moustakas *et al.* (1994) suggest that an increase in heavy metal contamination may affect the energy transfer from the light-harvesting complex to PS2. They also conclude that heavy metals affect the photosynthetic apparatus in multiple ways, by decreasing the quantum yield of PS2 photochemistry, reducing the effective and total Chl content, and inhibiting the enzymes of the Calvin cycle. Cadmium induces phytotoxic effects in a number of plant species. These effects can include inhibition of the Calvin cycle, decreases in quantum yield, and disassembly of PS2 proteins (Popovic *et al*. 2003). Effects due to copper and zinc followed a similar pattern to those for cadmium. Although both copper and zinc are essential micronutrients for metabolic purposes, toxic accumulation is a very real problem during exposure to high concentrations.

The R/FR ratio has been used to detect stress in other phytoremediation research. In studies of a range of instruments, Schuerger *et al.* (2003) found that the R/FR ratios were best used to detect a general stress response rather than to make any form of direct diagnosis. These studies found that plants with the lowest R/FR ratio were those grown at 0.5 g(Zn) m^{-3}. Plants grown at either deficient or elevated levels both showed higher fluorescence ratios. This ambiguous response may limit the effectiveness of a simple device such as the PHM which only measures fluorescence at two wavelengths.

A goal for phytoremediation studies is to encourage high biomass plants to take up high fluxes of metals into their shoots. The potential application of the PHM in this strategy is in optimising phytoremediation technology. Monitoring the health of the plants used could aid species selection and optimisation of soil treatment, concentration, time, and duration.

Fluorescence regions sensed: The wavelengths measured by the PHM are of interest since they are different to those used by other sensors. Other fluorescence sensors are based around the four ultraviolet radiation-induced fluorescence maxima described in Lichtenthaler and Miehé (1997) – 440 nm (blue, B), 520 nm (green, G), 690 nm (R), and 740 nm (FR). Studies have focused on four fluorescence ratios: B/G, B/R, B/FR, and R/FR. The types of stress and the plant's response mechanisms determine how these ratios change. Although changes in these ratios allow early stress detection, the changes do not directly identify the type of stress. Short-term stresses are thought to increase fluorescence due to disturbances to the photosynthetic apparatus. Longer-term stresses lead to the decline of the Chl content and thus a decline in measured levels of fluorescence. The R/FR ratio also increases as a result of a reduction of re-absorption of R fluorescence.

The wavelength used for excitation is an important feature of any monitor. Schweiger *et al.* (1996) found that the B/R and B/FR excitation spectra were most suitable for stress indication, and that both these ratios increased under the influence of stresses in a range of monocot and

dicot plants. Their work suggests that an excitation wavelength of between 355 and 390 nm would be favoured. This wavelength range permits excitation of B, G, R, and FR fluorescence bands and therefore allows detection of B/R and B/FR fluorescence ratios. The PHM uses B (420–460 nm), which is considered the most suitable when only R and FR fluorescence is measured. The latter exhibit broad maxima in this region (Schweiger *et al.* 1996).

The PHM senses fluorescence at 688 and 762 nm. Since the *in vivo* Chl fluorescence peak is at approximately 690 nm, many of the monitors measure fluorescence in this region. However, many of the other studies have looked at fluorescence between 730 and 740 nm to represent FR fluorescence (Lichtenthaler and Miehé 1997, Gitelson *et al.* 1999, Barócsi *et al.* 2000). This allows detection of the distinct fluorescence maximum at 740 nm displayed by dark green plant tissue. Measurement of fluorescence at 688 and 762 nm avoids the need for an interferometer or spectrometer. Thus *in situ* Chl fluorescence measurements can be taken outside the laboratory environment (Kebabian *et al*. 1999).

Most Chl fluorescence can probably be attributed to the activity of PS2 (Lichtenthaler and Miehé 1997). Schreiber (2003) describes how most fluorescence originates from the antenna pigments of PS2 and hence the intensity relates to the rate of charge separation of PS2. However, recent studies by Franck *et al.* (2002) have demonstrated that the relative contribution of PS1 varies at different wavelengths. The PS1 spectrum has a maximum at 722 nm and a shoulder at 680 nm. The PS2 maximum at 684 nm reduces the contribution of PS1 to overall fluorescence at this wavelength to less than 10 %. At wavelengths above 700 nm the contribution of PS1 is estimated to be >30 %. This pattern is thought to be true of most plant species. It may be that at 760 nm the contribution of PS1 to fluorescence could be >50 %. Plant stresses principally impact on PS2, and hence the main differences in the R/FR ratio may be a result of changes in fluorescence quenching at 690 nm (Freedman *et al*. 2002). Since there is overlap in the fluorescence spectra of PS1 and PS2 it is difficult to separate the contribution of each photosystem to overall fluorescence.

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