MINERAL DEFICIENCY STRESS

Effect of Phosphorus Deficiency on Reflectance and Chlorophyll Fluorescence of Cotyledons of Oilseed Rape (*Brassica napus* L.)

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Abstract

The spectroscopic changes in reflectance and fluorescence caused by phosphorus (P) starvation in *Brassica napus* L. young plants were evaluated. P deficiency produced an important decrease in reflectance values between 500 and 650 nm for both intact leaves and cotyledons. Furthermore, cotyledons under P deficiency showed a Chl-F ratio in the red/far-red region ($F_{red}/F_{far\text{-}red}$) lower than that of non-stressed plants (1.91 and 2.89 respectively). As minimal differences in $F_{red}/F_{far\text{-}red}$ were detected in leaves, P deficiencies may be better perceived by measuring changes in Chl-F emission in cotyledons than in leaves. Stressed cotyledons also showed different emission spectra in the blue green (maxima at 470 and 560 nm) from those of non-stressed cotyledons. The results are explained in terms of higher anthocyanin and chlorophyll contents and of damage to photosystem II. We evaluate that measuring variations in fluorescence and reflectance data may be useful to detect early damages induced by P stress.

Introduction

Oilseed rape is an important crop for the production of high quality food oil, animal feed and biodiesel. Compared with other oil crops, oilseed rape has a larger demand of plant nutrients, including phosphorus (P) (Grant and Bailey 1993). Although P is one of the most unavailable and inaccessible macronutrients required by plants, it plays a key role in an array of plant processes (Vance et al. 2003). P is a primary substrate of photosynthesis and has structural functions in membranes (Iglesias et al. 1993). It also regulates the whole energetic metabolism of plants due to its presence in ATP, ADP, AMP and pyrophosphate molecules (Xu et al. 2007) and participates in signalling, enzyme activation-deactivation, and respiration processes (Vance et al. 2003). Phosphorus starvation leads to changes in morphological, physiological and biochemical processes, such as a decrease in the root/shoot ratio, modifications in root architecture, accumulation of anthocyanins and a delay in plant ripeness (Rodriguez et al. 1994, Rubio et al. 2003, Vance et al. 2003, Rubio and Lynch 2007).

Stress in plants may also be manifested as a change in their optical and spectroscopic properties (Neuner and Larcher 1990, Ratinam et al. 1994, Kościelniak and Biesaga-Kościelniak 1999, Rapacz et al. 2008). These variations can be used as stress-indicative tools, relevant in field application because of their potential use in remote sensing. In this context, reflectance and fluorescence spectroscopies may provide a rapid, non-destructive diagnostic method for detecting and quantifying environmental stresses and damage to the photosynthetic apparatus of leaves, thus allowing testing these analytical procedures on intact biological material (Mino et al. 1998, Merzlyak et al. 2003, Papageorgiou 2004).

In the red and far-red regions of the electromagnetic spectrum, fluorescence emission in plants is mainly caused by chlorophyll-*a*. This fluorescence is characterized by two bands (a red band at about 687 nm and a far-red

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band at about 737 nm). Under conditions of low photon flux impinging on the leaf, all the electron acceptors in the photosynthetic chain are open and the fluorescence spectrum obtained under these conditions is called initial fluorescence (F_0). This spectrum is obtained when dark-adapted plant materials are irradiated with low light intensity to prevent the induction of the variable portion of the Chl-fluorescence. The $F_{red}/F_{far\text{-}red}$ fluorescence ratio obtained from these spectra is a parameter that has been correlated with different stress factors (Lichtenthaler and Rinderle 1988, Agati et al. 1993, 2000, Subhash et al. 1999).

Plant leaves also emit in the blue-green portion of the electromagnetic spectrum. The blue fluorescence is characterized by a peak at about 450 nm and the green fluorescence by a maximum at about 530 nm. As previously reported (Lang et al. 1991), this emission is mainly originates in the cell-wall. Phenolic substances such as chlorogenic acid, caffeic acid, coumarins and stilbenes may be responsible for the blue fluorescence emission, whereas substances like berberine and quercetin are responsible for the green fluorescence (Lang et al. 1991).

The main purpose of our work was to evaluate the effects of P depletion on the spectroscopic properties of leaves and cotyledons and to assess fluorescence emission and reflectance as indicators of premature stress in oilseed rape plants.

Although the spectroscopic analysis of cotyledons has been scarcely studied (Vertucci et al. 1985, Lebkuecher et al.1999), it seems to be helpful to detect P-induced stress at an early developmental plant stage.

On the other hand, it is known that P starvation leads to destruction of PSII in different plants (Jacob and Lawlor 1993). In this context, the second aim of this work was to evaluate the possibility of detecting PSII damage through the interpretation of chlorophyll-fluorescence spectra. For this purpose, we applied a theoretical model to account for light re-absorption processes affecting experimental data. To our knowledge, this is the first time that this kind of theoretical approach is applied to the fluorescence spectra of a useful crop for food industry.

Materials and Methods

Plant growth conditions

Seeds of oilseed rape *Brassica napus* L. were surface disinfected by soaking in 3% NaOCl for 10 min and rinsed three times with sterile distilled water. Disinfected and pre-germinated seeds (2 days at 25 °C in the dark) were transferred to glass tubes containing 20 ml of a sterile mineral solution as described by Murashige and Skoog (Murashige and Skoog 1962), either with (+P) or without (−P) potassium phosphate. Potassium concentration was kept at a constant level by adding KCl. A filter paper with an inverted U shape was previously inserted into the glass tube as mechanical support of the seedlings. Plants were grown in an orbital shaker (Vicking Shaker pro; Vicking S.A., Buenos Aires, Argentina) at 140 rpm, placed in a plant growth chamber and kept at a 16 h light-8 h dark cycle, in which light was emitted at a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} using Philips TLD 865 fluorescent tubes (Philips B.V., Amsterdam, the Netherlands) and at a temperature of 22 ± 2 °C. Plants were grown until the first leaves were completely extended (around 30–35 days after transplanting).

Pigment content determination

Chlorophyll-*a*, chlorophyll-*b*, carotenoids and anthocyanins were determined both in leaves and cotyledons. About 0.1–0.2 g weight of biological tissue (one whole cotyledon or leaf) was placed in a mortar half full with liquid nitrogen and it was ground to powder. Then, pigments were extracted from the powdered sample by adding 2.0 ml of the extraction solvent 85% acetone and 15% Tris stock buffer (1% w/v Tris final concentration; adjusted to pH 8 with HCl) previously cooled in ice. The extract was centrifuged at 12 000 ***g*** for 3 min. A defined quantity of supernatant (1 ml) was removed and diluted to 3.0 ml. Its absorbance was measured at 537, 663, 647 and 470 nm in a 1-cm path-length cell. The pigment content was then calculated according to Eqns (1–4) (Sims and Gamon 2002).

$$\text{Anthocyanin} = 0.08173A_{537} - 0.00697A_{647} - 0.002228A_{663} \quad (1)$$

$$\text{Chl}_a = 0.01373A_{663} - 0.000897A_{537} - 0.003046A_{647} \quad (2)$$

$$\text{Chl}_b = 0.02405A_{647} - 0.004305A_{537} - 0.005507A_{663} \quad (3)$$

$$\text{Carotenoids} = \frac{(A_{470} - (17.1(\text{Chl}_a + \text{Chl}_b) - 9.479 \times \text{Anthocyanin}))}{119.26} \quad (4)$$

where A_x is the absorbance of the extract solution in a 1-cm path-length cuvette at wavelength x.

The units for all the equations are μmol ml^{-1}. Eqns (1–4) were deduced in reference (Sims and Gamon 2002) for different plant species to assess pigment content in the presence of high amounts of anthocyanins.

Reflectance measurements

Diffuse reflectance was measured as a function of wavelength from 300 to 800 nm for groups of stacked cotyledons [$R_\infty(\lambda)$, diffuse reflectance for an optically thick sample displaying zero transmittance]. Diffuse reflectance measurements were performed by means of a spectrophotometer (UV3101PC; Shimadzu, Tokyo, Japan) equipped with an integrating sphere (ISR-3100; Shimadzu) (Scheme 1). Barium sulphate was employed as a white reference standard to adjust 100% reflectance level. The baseline correction was performed with barium sulphate placed in the two positions of the sphere as shown in Scheme 1. After this correction, the sample-side standard white plate was exchanged by the set of packed cotyledons or leaves to record diffuse reflectance. The light-incidence angle to the reflecting sample was set to zero degree so that the specularly reflected light was removed from the entrance window and only the diffuse part of the reflectance was measured. The area sampled was 2.54 cm^2.

The remission function (Wendlandt and Hecht 1966) F(R) was obtained as a function of wavelength (λ) from diffuse reflectance data recorded for the group of stacked cotyledons according to eqn (5).

$$F(R) = \frac{(1 - R_\infty(\lambda))^2}{2R_\infty(\lambda)} \quad (5)$$

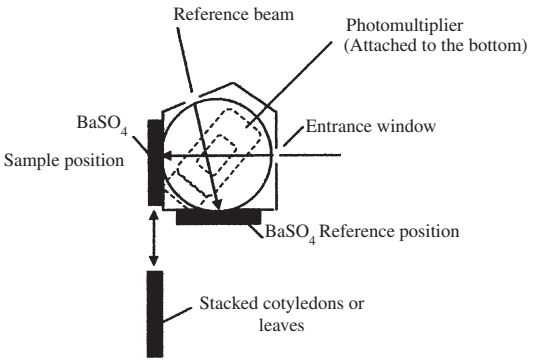
This quantity is needed for calculations used in the theoretical approach to correct distortions in fluorescence emission spectra due to light re-absorption.

Fluorescence measurements

Fluorescence measurements were performed using a steady-state spectrofluorometer (QuantaMaster, PTI – Photon Technology International, Brunswick, NJ, USA). Measurements on the adaxial faces of leaves and cotyledons were carried out in front face geometry. Fluorescence spectra were recorded for intact leaves or cotyledons superimposed in groups of three or four so that no light was transmitted through them. Excitation wavelength was set to 460 nm for the red/far-red emission region and to 340 nm for the emission in the blue-green section. Emission spectra were recorded from 600 to 800 nm (at excitation wavelength 460 nm) and from 400 to 600 nm (at excitation wavelength 340 nm) and corrected by the detector response to different wavelengths. Fluorescence spectra were obtained as the number of counts recorded by the spectrofluorometer photomultiplier (810, PTI – Photon Technology International) as a function of wavelength. The photomultiplier used allowed digital (photon counting) detection. Excitation photon flux in emission experiments was lower than 20 μmol m^{-2} s^{-1} in all cases, so that all electron acceptors in the photosynthetic unit were open and no induction of variable fluorescence kinetics was present. It was further confirmed that the selected slit led to invariable fluorescence distribution upon repetition of fluorescence spectra.

As a consequence, all the electron acceptors could be considered open and the measured fluorescence corresponded to the ground emission F_0. To attain these conditions, an excitation slit of 0.5 nm was used. The whole set of measurements on fresh leaves and cotyledons was performed immediately after their excision from the plants. The influence of fluorescence emission on reflectance data was neglected in all cases (Zarco-Tejada et al. 2000, Cordon and Lagorio 2006).

Cotyledons and leaves were dark-adapted for 15 min before recording each fluorescence spectrum. All measurements were performed at room temperature (about 25 °C). The area sampled was 1 cm^2.



Scheme 1 Integrating sphere attachment; Shimadzu ISR-3100.

Correction for light re-absorption processes

To obtain fluorescence emission corrected by light re-absorption processes [$I_f^c(\lambda)$],fluorescence spectra corrected by the detector spectral response [$I_f^e(\lambda)$]were treated as indicated in eqns (6) and (7) (Lagorio et al. 1998, Ramos and Lagorio 2004, Cordon and Lagorio 2006),

$$I_f^c(\lambda) = \frac{I_f^e(\lambda)}{\gamma(\lambda, \lambda_0)} \quad (6)$$

$$\gamma(\lambda, \lambda_0) = \frac{1}{1 + \sqrt{\frac{F(R_\lambda)}{F(R_\lambda)+2}}} \quad \frac{1}{1 + \sqrt{\frac{F(R_\lambda)(F(R_\lambda)+2)}{F(R_{\lambda_0})(F(R_{\lambda_0})+2)}}} \quad (7)$$

where I_f stands for the fluorescence emission intensity (in arbitrary units), F(R) is the remission function

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defined by eqn 5 and λ and λ_0 denote emission and excitation wavelengths respectively.

This model was primarily deduced for correcting light re-absorption processes in systems of dyes adsorbed in powders (Lagorio et al. 1998) and was then extended to plant leaves. The correction procedure used is based on a physical description of the interaction between light and materials following the general basis of the Kubelka–Munk theory (Wendlandt and Hecht 1966). Consequently, this approach consists of a two-flux model based on the following assumptions: (i) the leaves behave as an ideal diffuser, (ii) emission is produced in each volume element and it is decomposed into two-photon flows having the same magnitude but opposite directions, (iii) the sample is so thick that no light is transmitted through it. To confirm the last assumption, a group of stacked leaves or cotyledons was used in the present study. The model has been previously validated on leaves by comparing the fluorescence spectra of stacked leaves corrected for re-absorption processes with the fluorescence spectra of a thin layer of chloroplasts (Ramos and Lagorio 2004). So, the resultant spectra after the application of this correction function may be considered as the spectra that would be obtained for a thin layer of chloroplasts (free from re-absorption artifacts) from the tested plant material.

In general, values for the gamma function $\gamma(\lambda, \lambda_0)$ may start from one (when no re-absorption is present) and decrease towards zero when re-absorption becomes more important. This correction function depends on the excitation wavelength because the absorption properties of leaves at the irradiation wavelength determine the depth of light penetration and consequently the path length available for the emission to be re-absorbed. In fact, when absorption at excitation wavelength is high, the depth of light penetration is low, fluorescence is produced at the layers next to the leaf surface and fluorescence re-absorption is scarce. Inversely, when absorption at excitation wavelength is low, the depth of light penetration is high, fluorescence is generated from the chlorophyll molecules inside the leaf tissue and it is further re-absorbed in its way out of the leaf.

Results

Measurements of pigment content

Pigment content of leaves and cotyledons from both stressed and control oilseed rape plants are presented in Table 1. P stress increased total content of chlorophyll, carotenoids and anthocyanins in both leaves and cotyledons. The chlorophyll *a*/*b* ratio was not significantly altered.

P starvation decreased shoot and increased root dry weights (Table 2) leading to an increase in the root/shoot ratio.

Reflectance spectra

The most prominent effect of P on reflectance spectra in leaves (Fig. 1) and cotyledons (Fig. 2) was the decrease ($P < 0.0005$, Tukey's test for leaves and cotyledons) in reflectance values at 500–650 nm for P-depleted plants.

The level of P supply did not significantly affect the reflectance in the red region. In the far-red interval, reflectance was lower for P-depleted leaves and higher for P-depleted cotyledons.

Fluorescence emission spectra

Phosphorus did not show significant effects on the fluorescence emission spectra of oil rape leaves (results not shown). In contrast, significant changes were detected in cotyledons. Results for emission in the red-far red region are shown in Fig. 3, where the average experimental

Table 1 Pigment content in both stressed and control cotyledons and leaves

Pigment content (μmol g^{-1} fresh weight)	Cotyledons		Leaves	
	−P	+P	−P	+P
Chl *a*	0.36 ± 0.06**	0.15 ± 0.02**	0.62 ± 0.07*	0.31 ± 0.04*
Chl *b*	0.17 ± 0.02**	0.08 ± 0.01**	0.25 ± 0.03*	0.14 ± 0.02*
Total Chl	0.53 ± 0.08**	0.23 ± 0.02**	0.87 ± 0.09*	0.46 ± 0.06*
Chl *a*/*b* ratio	2.03 ± 0.11 NS	1.97 ± 0.05 NS	2.45 ± 0.18 NS	2.19 ± 0.07 NS
Carotenoid	0.30 ± 0.02***	0.15 ± 0.02***	0.41 ± 0.04*	0.24 ± 0.03*
Anthocyanin	0.24 ± 0.05*	0.08 ± 0.03*	0.29 ± 0.09*	0.07 ± 0.0013*

Statistical analysis was performed using ANOVA. All data represent the means ± S.E. (from at least three independent series of experiments).

*,**,***Degree of statistical significance, <0.05, 0.01, and 0.001, respectively; and NS, not significantly different (Tukey's test).

+P, control plants and −P, plants grown under phosphorus depletion.

Table 2 Shoot and root dry weight (mg) in stressed and non-stressed oilseed rape plants

	+P (mg)	−P (mg)
Shoot	59.63 ± 2.20**	21.83 ± 1.30**
Root	5.12 ± 0.40**	10.21 ± 0.30**
Root/shoot	0.09 ± 0.01**	0.45 ± 0.03**

Statistical analysis was performed using ANOVA. All data represent the means ± S.E. (from at least three independent series of experiments).
**Degree of statistical significance [<0.01 (Tukey's test)].
+P, control plants and −P, plants grown under phosphorus depletion.

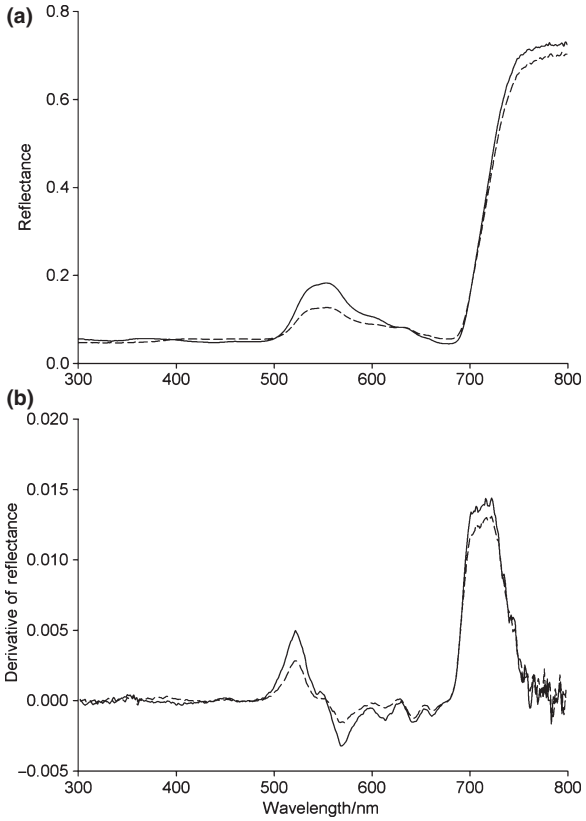

Fig. 1 (a) Average reflectance spectra for P-stressed (- - -) and control (——) leaves. Each curve corresponds to averages from five reflectance determinations. (b) First derivatives of reflectance spectra for P-stressed (- - -) and control (——) leaves.

fluorescence emission spectra for five samples corrected by the detector response are shown. Spectra were normalized at 737 nm to allow a better comparison between the spectral distributions. The experimental fluorescence ratio (F_{687}/F_{737}) was higher for control than for stressed samples. As deviations from average experimental values of F_{687}/F_{737} were lower than 20% in all cases, the differences

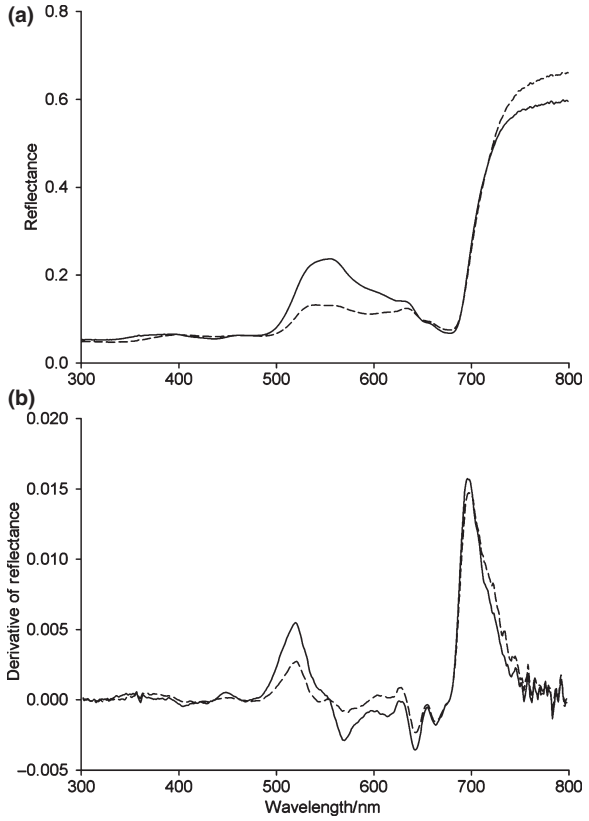

Fig. 2 (a) Average reflectance spectra for P-stressed (- - -) and control (——) cotyledons. Each curve corresponds to averages from five reflectance determinations. (b) First derivatives of reflectance spectra for P-stressed (- - -) and control (——) cotyledons.

observed upon stress induction were significant according to *t*-test $P < 0.05$.

Average experimental emissions in the blue-green region obtained from cotyledons at an excitation wavelength of 340 nm are shown in Fig. 4.

The blue-green fluorescence measured at 560 nm was significantly lower in cotyledons from P-stressed plants than in those from control plants. The dispersion of the data was ample, especially for stressed plants. The F_{470}/F_{560} ratio showed a deviation from the average values of 14% for control and 45% for P-stressed cotyledons. Neither stressed nor non-stressed leaves showed significant differences in their fluorescence emission spectra (data not shown).

Application of a theoretical model to account for light re-absorption processes

Correction of the experimental fluorescence emission spectra for light re-absorption processes was performed

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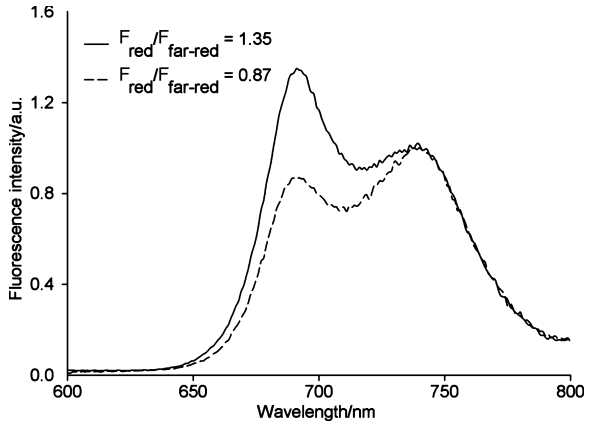

Fig. 3 Average experimental fluorescence emission spectra in the red/far-red for both P- stressed (- - -) and non-stressed (——) cotyledons, normalized at 737 nm and corrected by the detector response. Excitation wavelength: 460 nm.

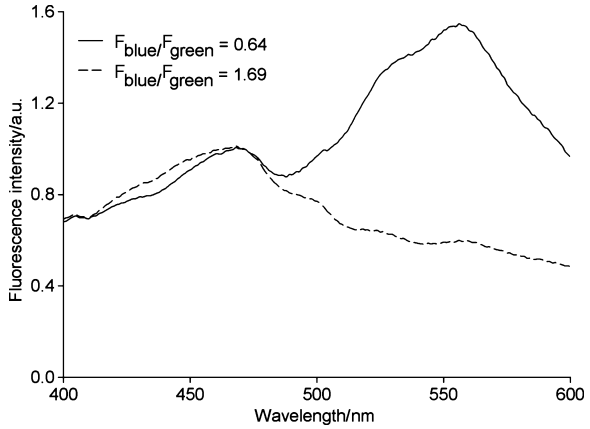

Fig. 4 Average experimental fluorescence emission spectra in the blue-green for both stressed (- - -) and non-stressed (——) cotyledons, normalized at 470 nm and corrected by the detector response. Excitation wavelength: 340 nm.

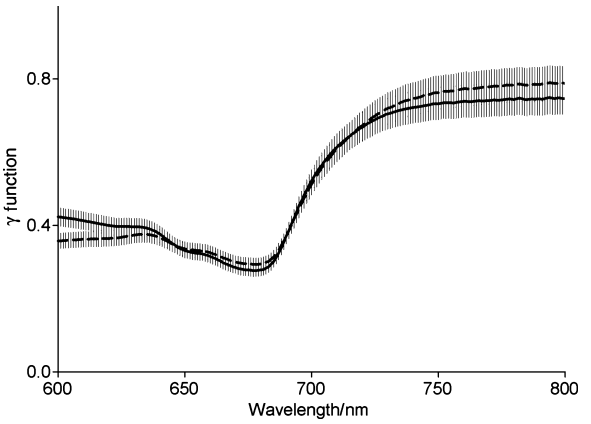

Fig. 5 Gamma function for both stressed (- - -) and non-stressed cotyledons (—) as a function of emission wavelength (600–800 nm), calculated for an excitation wavelength: 460 nm. The shaded area shows the standard deviation of measurements.

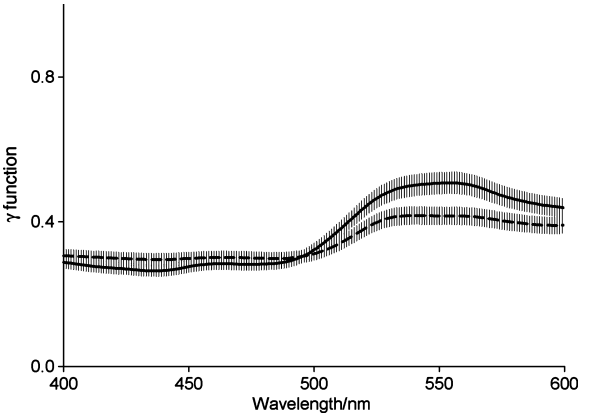

Fig. 6 Gamma function for both stressed (- - -) and non-stressed cotyledons (—) as a function of emission wavelength (400–600 nm), calculated for an excitation wavelength: 340 nm. The shaded area shows the standard deviation of measurements.

by calculating the Gamma function (eqn 7) and then dividing the experimental fluorescence spectra by this correction function (eqn 6).

A detailed analysis of the correction gamma function (eqn 7) for the red region (see Fig. 5) showed low values (corresponding to high re-absorption) between 600 and 700 nm and higher values at longer wavelengths.

The gamma function used to correct fluorescence spectra in the blue-green region showed low values (high re-absorption) in the whole interval (Fig. 6). Differences between control and stressed cotyledons for wavelengths shorter than 500 nm were within the experimental error.

When applying these correction functions to experimental data, spectra for the red/far-red and blue-green regions were obtained (Figs 7 and 8 respectively).

Spectra corrected by light re-absorption in Fig. 7 showed that the $F_{red}/F_{far\text{-}red}$ fluorescence ratio remained even lower for stressed cotyledons than for controls (differences are statistically significant at $P < 0.05$ and they cannot be attributed to experimental error). Differences in corrected spectra are also found in the blue-green region (Fig. 8) where fluorescence intensity at 550 nm is relatively less important for stressed cotyledons.

Discussion

The pigments analysis showed that P starvation led to an increase in chlorophylls, carotenoids and anthocyanins, both in leaves and cotyledons.

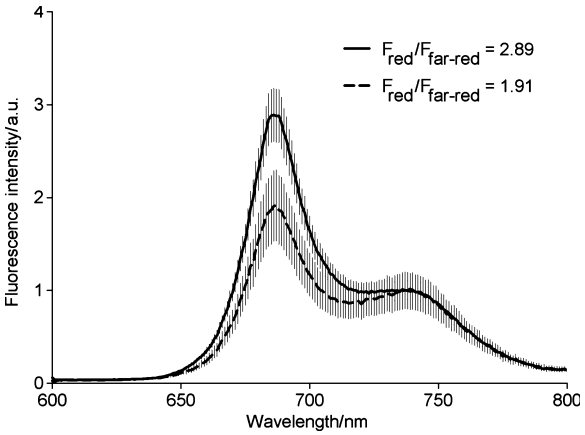

Fig. 7 Average fluorescence emission spectra in the red/far-red, corrected by light re-absorption processes, for both stressed (- - -) and non-stressed cotyledons (—). The shaded areas show the standard deviation of the results.

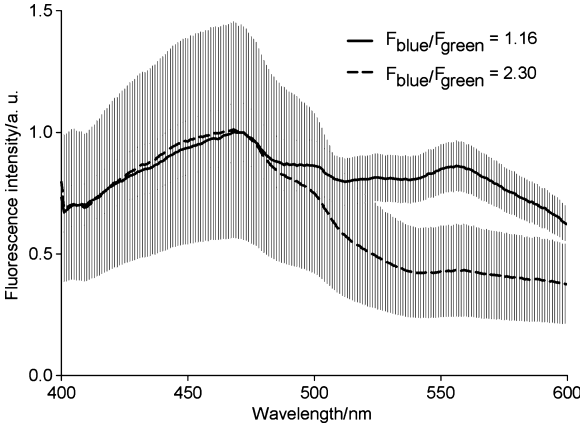

Fig. 8 Fluorescence emission spectra in the blue-green region, corrected by light re-absorption processes, for both stressed (- - -) and non-stressed cotyledons (—). The shaded areas show the standard deviation of the results.

The increase in anthocyanins is a symptom in several P stress processes (Close and Beadle 2003, Jiang et al. 2007). However, the biosynthesis of these pigments is induced by many other stresses such as visible and UVB radiation, drought, cold temperature, nitrogen deficiency, low pH and pathogen infection (Bergmannn 1992, Trull et al. 1997, Chalker-Scott 1999). As a consequence, their accumulation should not be considered as a specific response to P starvation, but as an indirect or secondary response (Trull et al. 1997). As a general rule, anthocyanins are considered light attenuators and antioxidants. In this context, it is believed that under stress situations, their main function is the quenching of the reactive oxygen species generated by stress (Neill and Gould 2003).

The results obtained for the root/shoot ratios in *Brassica napus* L. were similar to those obtained with other crops like *Helianthus annus* L. (sunflower) (Plesnicaronar et al. 1994) and *Arabidopsis thaliana* where it has already been shown that low phosphate in the medium inhibits plant growth rate and increases root proliferation (Jiang et al. 2007). The observed changes in the allocation patterns between above and belowground organs as affected by P status are very well known. When mineral elements, such as phosphorus, are scarce, plants often allocate a greater proportion of their biomass to the root system. This acclimatization response is a consequence of metabolic changes in the shoot and an adjustment of carbohydrate transport to the root (Hermans et al. 2006).

The reflectance spectra for both leaves (Fig. 1a) and cotyledons (Fig. 2a) at wavelengths between 300 and 500 nm did not show significant differences between control and stressed plants. In fact, in this region, chlorophyll-*a*, chlorophyll-*b* and carotenoids display such a high absorption that the reflectance signal is probably saturated and differences in pigments concentrations are not reflected in the spectra. The analysis of first derivatives (Gitelson and Merzlyak 1994) in this spectral region has also shown no important differences between stressed and control plants (Figs 1b and 2b).

We found important differences, however, between control and stressed plants for both leaves and cotyledons in the 500–600 nm region. As anthocyanins typically absorb at around 530 nm, the higher anthocyanin content in stressed plants (Table 1) led to higher absorption and lower reflectance in the green region (Figs 1a and 2a). The effect was most pronounced for cotyledons than for leaves. Anthocyanins are responsible for the reddish colour observed in the stressed leaves and cotyledons in our work. As previously observed by Neill and Gould (Neill and Gould 1999), the red color is not due to higher reflectance in the red region but to the subtraction of green wavelengths from the reflected light. First derivatives of reflectance in this region also presented a different behaviour between control and stressed species. In this case, even when maxima and minima positions remained the same, the derivative amplitude resulted lower for the stressed plants in all the cases. Derivatives sign showed an important change (from positive to negative) near 553 nm.

From 600 to 700 nm, reflectance properties are determined by the chlorophyll content. The higher chlorophyll content in stressed plants was manifested as a decrease in reflectance at 600–650 nm, mainly for cotyledons. From 650 to 700 nm, neither leaves nor cotyledons showed significant differences. The high absorbance around 680 nm induced by chlorophyll concentrations produce saturation of the reflectance signals and therefore prevents the detection of any difference in the pigment concentration.

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The slopes of the derivatives were very high from 680 to 700 nm, but they could not be considered as a differential feature between stressed and non-stressed plants. In the far-red region, over 700 nm, reflectance is usually determined by the material structure (Slaton et al. 2001) and differences in this region may not be related to pigment concentration. In the case of cotyledons, where the differences were more important, the higher reflectance in P-depleted plants might be because stressed cotyledons were thicker and spongier than control ones. In conclusion, in reflectance spectra, values in the region 530–555 nm for cotyledons showed the most sensitive response to P stress. To characterize reflectance data further, the absorption depths were calculated following Run-he-Shi et al. 2006 at 680 nm. In leaves, the obtained absorption depths values were 0.087 and 0.114 for control and stressed plants respectively. In cotyledons, values were very similar: 0.150 for control and 0.166 for P depleted plants. The similarity in absorption depths at 680 nm is not surprising as small differences in the absorption bands at this wavelength may be observed in the reflectance spectra. The use of absorption depth at 680 nm would not be a good parameter to evaluate P deficiency. Taking into account the increase in anthocyanins content during stress and its manifestation in reflectance spectra, one would expect the absorption depth near 553 nm to be sensitive to the stress condition. However, the fact that a maximum of reflectance was detected at around 553 nm, did not allow the absorption depth to provide a reliable parameter.

On the other hand, the fluorescence ratio in the red (F_{680}/F_{737}) (Fig. 3) and the fluorescence ratio in the blue-green region (F_{blue}/F_{green}) (Fig. 4) from the experimental emission spectra of cotyledons also turned out to be sensitive tools as they changed upon stress from 1.35 to 0.87 and from 0.64 to 1.69 respectively. Unlike that observed in cotyledons, leaves did not show significant differences. Actually, cotyledons are one of the parts of the plants earlier affected by P starvation, as they contain the major P store as phytic acid, which is subsequently converted to inorganic P by phytases and translocated to the plant (Mitchell and Allsopp 1984). It has been previously shown that different organs in the same plant may differ in their sensitivity to a given stress (Yusufov and Alieva 2002). These experimental facts support the decision of including cotyledons in our work with the aim of detecting stress as early as possible.

The variation in fluorescence spectra caused by stress is very clear and significant, even though the interpretation of the factors leading to this change is not so straightforward.

To analyse the red emission, the following points should be taken into account: (i) under our experimental conditions (low excitation-photon flux and room temperature), both photosystems PSI and PSII emit spectra, (ii) PSII is responsible for emission at 680 nm, whereas PSII and PSI are responsible for emission at 737 nm and (iii) PSI contribution at 737 nm can be higher than 50% (Agati 1998, Pfundel 1998). Additionally, the spectral distribution for the fluorescence emission is distorted by light re-absorption processes (Ramos and Lagorio 2004). So, changes in the experimental red/far-red fluorescence ratio may be caused even by differences in light re-absorption of fluorescence emission or by variations in the emission of one photosystem relative to the other. As the emission peak at 687 nm overlapped with chlorophyll absorption spectra, the decrease in the F_{680}/F_{737} fluorescence ratio (Fig. 3) is consistent with the higher chlorophyll content in cotyledons of P-stressed plants. However, another alternative for the observed ratio decrease may be due to the damage of PSII which is well documented in literature when P stress was induced (Jacob and Lawlor 1993). To separate both factors, experimental spectra should be corrected by light re-absorption processes so that any remaining difference could be attributable to the different contribution of each photosystem to emission. To account for re-absorption artifacts, there are three correction models in literature (Cordon and Lagorio 2006) and there is still controversy about their applicability. One of these methods (GM in reference Cordon and Lagorio 2006) is based on empirical considerations and measurements of the transmittance and reflectance of a single leaf allows the calculation of a correction function independently of the excitation wavelength and it usually gives high values for the corrected fluorescence ratio ($F_{red}/F_{far\text{-}red}$) close to the ratio found for chlorophyll-*a* in solution (where no photosystems are present). The other two methods (AM and LM in reference Cordon and Lagorio 2006), based on independent physical approaches, give results similar to each other. While one of them (AM) needs the reflectance and transmittance from a single leaf to estimate the correction function, the second one (LM) needs the reflectance of a thick layer of leaves. Both methods use correction functions that depend on the excitation wavelength and they give lower values for the corrected fluorescence ratio than GM.

Owing to the small cotyledons size and the large measuring area in our spectrophotometer, transmittance and reflectance could not be measured on a single cotyledon. Reflectance measurements, instead, could be perfectly performed on a thick layer of stacked cotyledons.

Due to the reasons presented above, we decided to use our own model (LM), which was previously validated by proving the coincidence between the corrected spectra of intact leaves and those originated from a thin layer of chloroplasts where no re-absorption

occurred (Ramos and Lagorio 2004). The gamma function calculated for the correction of the red and far-red emission showed that gamma values at 680 nm were as low as 0.28 (Fig. 5). This result shows the importance of the re-absorption process at 680 nm, where the emission intensity has to be multiplied by a factor of about 3.6 (1/0.28) to produce the corrected value. The emission band at 737 nm, on the other hand, was affected by a much lower factor (about 1.4). This is coherent with the fact that overlapping between pigment absorption and chlorophyll emission was higher for the 680 nm band.

When we compared P-stressed and non-stressed cotyledons, the first relevant feature was the lower value for the gamma function in stressed cotyledons in the region between 600 and 650 nm. This result may be explained by the higher proportion of anthocyanins and chlorophylls for stressed cotyledons. However, gamma values were similar for wavelengths longer than 650 nm within the experimental error. We consider that this result may be explained by the high absorption of the sample at the excitation wavelength, and the subsequent low light penetration. After application of the model to the data in Fig. 3, there were still differences in the fluorescence ratio. This result is consistent with possible damage to PSII occurring during the stress process. Damage to PSII has been reported for sunflower and maize grown under phosphate deficiency (Jacob and Lawlor 1993) and has also been identified in many stress processes, being very sensitive to the environment of the plant including its nutrition status. In fact, PSII has been identified as the main target for the photoinhibitory damage when plants are submitted to hard stresses (Chow et al. 1989). More specifically, it has been proven that P starvation leads to PSII photoinhibition (Jacob and Lawlor 1993). Deo and Biswal (2001) have found that water deficiency also induces PSII damage in cotyledons.

Chlorophylls *a*/*b* ratio was the same for both stressed and non-stressed cotyledons. This fact agrees with the observed reduction in PSII upon stress, as it has been shown that for cotyledons PSII activity may decrease while chlorophylls *a*/*b* ratio is kept almost constant (Ghosh et al. 2001).

Our analysis of the experimental emission spectra in the blue-green region showed a relative reduction at the 560 nm region that might be explained by the presence of high amounts of anthocyanins in stressed cotyledons. Their absorption spectra present their maxima at 500–600 nm (Moreira et al. 2003), thus overlapping with the fluorescence emission in this region.

The correction using the gamma function was also calculated for the blue-green emission (Fig. 6). For wavelengths longer than 500 nm, the correction function was lower (higher re-absorption) for the stressed plants in agreement with their higher content of pigments (particularly anthocyanins). Below 600 nm, differences in the gamma function were smaller.

The corrected F_{470}/F_{560} fluorescence ratio became even higher for stressed cotyledons (significantly different, $P < 0.005$ according to the *t*-test). This fact seems to indicate that differences in anthocyanin content do not account entirely for the differences in experimental spectra.

The present study indicates that P starvation may be early detected by an important reflectance decrease in values at 500–600 nm for both cotyledons and leaves. The effect is more pronounced for cotyledons than for leaves. In spite of that, separation of cotyledons from leaves in remotely sensed images will be impossible and the extra sensitivity of cotyledons might be profited at small distance in a field detection level, but not from a satellite monitoring. Fluorescence is also, in this case, not suitable for remote sensing in leaves as no differences in fluorescence were observed. It may be, however, a sensitive tool at a field level when using cotyledons and it has the advantage over reflectance, providing information not only on stress but also on photosystem damage in the plant, when the red and far-red regions were analysed.

It should be noted that for practical applications, the use of a correction model to account for re-absorption was not necessary as the changes represent variations within the experimental data. However, the use of models that allow the 'subtraction' of the light re-absorption process has interesting consequences in providing additional information on physiological aspects caused by stress (Cordon and Lagorio 2006). As this relevant feature is still under discussion in the literature, the results obtained here from the application of a theoretical model to obtain the fluorescence emission free from artifacts due to re-absorption may be considered as a preliminary work on the subject. Future work on this last area is necessary to continue exploring the theoretical interpretation of fluorescence data.

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