In-field assessment of wheat-leaf polyphenolics using the new optical leaf-clip Dualex

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Abstract

In addition to the widely known leaf chlorophyll (Chl) detection by absorbance or reflectance, we propose here to use optical assessment of leaf polyphenolics content (Phen) as an indicator of crop nitrogen (N) status. We developed a portable instrument based on chlorophyll fluorescence excitation spectra, the Dualex®, adapted to measurements of leaf Phen content in the field. It operates in full daylight with a UV beam at 375 nm and a red reference beam at 650 nm. Thanks to the use of a patented feedback loop, measurements take less than a second. We used the Dualex® to follow the dynamics of wheat-leaf Phen in fields that had received different N fertilisation rates. We showed that wheat crops under N deficiency accumulate more Phen. Thus, Phen responded in an opposite manner to Chl. We propose the use of the Chl to Phen ratio as an indicator of crop N status. This new indicator is more sensitive than Chl or Phen alone, and alleviates, at least partially, the problem of gradients along wheat leaves. This is an important argument in favour of the implementation of both Chl and Phen measurements to the next generation of active non-contact sensors for real-time site-specific N management.

Keywords: optical sensors, leaf chlorophyll fluorescence, epidermal ultraviolet absorbance, nitrogen fertilisation, wheat.

Introduction

Optical indicators at the leaf level

Optical techniques are currently the only satisfactory means of making non-destructive and rapid *in situ* assessments of crop Chlorophyll (Chl), N and protein statuses. Optical leaf-clips are useful tools in achieving these goals. These can be used to validate relationships between agronomic traits and optical signals, and thus aid in the design of optical indicators for decision support systems (DSS). Chlorophyll leaf-clips have been used in this way to validate reflectance-based assessments of crop Chl content, but they are also implemented as stand alone techniques for N management. For example, the Hydro N-tester leaf-clip, derived from the SPAD-502 meter (Minolta, Japan) and commercialised by Yara, is used in wheat DSS linked to the third and forth N side dressing (Hoel, 2002; Lopez-Bellido *et al.*, 2004). The mean of 30 random measurements of uppermost leaf Chl content gives good predictions of crop N status provided calibrations are made for soil type and wheat variety. The accuracy of such Chl estimations of crop N status can be increased by taking account of leaf mass per unit area (LMA) (Peng *et al.*, 1993). Unfortunately, LMA measurements are destructive and therefore not applicable to precision agriculture. Alternatively, non-destructive measurements of leaf polyphenolics (Phen) could be used, since Phen is known to be related to LMA (Lovelock *et al.*, 1992; Goulas *et al.*, 2004).

Polyphenolics as a new optical indicator

Leaf Phen has recently been proposed as a surrogate indicator of crop N status (Cartelat *et al.*, 2005). These are a family of compounds that play a large variety of roles in the plant, from UV- and oxidative stress-protection to defence against pathogens and herbivores (cf. Dixon & Paiva, 1995; Jones & Hartley, 1999). In wheat leaves, the major UV absorbers are derivatives of the flavonoid isoorientin and caffeic acid (Cartelat *et al.*, 2004). Because the UV light source of the Dualex is set at 375 nm, the device preferentially detects the presence of isoorientin derivatives (Figure 1). The potential of leaf Phen to be used as indicators of crop N availability stems from the Protein Competition Model (PCM) of polyphenolic allocation (Jones & Hartley, 1999). This mechanistic model specifies that under N shortage, a larger proportion of newly assimilated carbon will be allocated to non N-containing polyphenolics used for plant structure and defence.
Phen absorption spectra all have maxima in the UV region (Cerovic *et al.*, 2002) (cf. Figure 1). Because of very low reflectance (less than 5%) and an almost zero transmittance of leaves in the UV (Gates *et al.*, 1965), classical spectroscopic methods cannot be easily applied. This is why alternative methods based on screening of Chl fluorescence (ChlF) excitation have been developed (Bilger *et al.*, 1997; Ounis *et al.*, 2001; Goulas *et al.*, 2004). A UV beam, absorbed by the epidermis, is used to excite the Chl in the leaf mesophyll. It is compared to a visible beam for which the epidermis is transparent, and thus acts as a reference. The ratio of the ChlF emitted under this dual excitation is proportional to the absorbance of the epidermis in the UV. This UV absorbance gives the concentration of Phen in the epidermis. Because the majority of Phen is present in the epidermis, this measurement can be used to estimate the overall leaf Phen content (Goulas *et al.*, 2004).
In the present study, we tested the sensitivity of Phen to wheat N nutrition, and compared Chl and Phen as optical indicators of wheat N status.

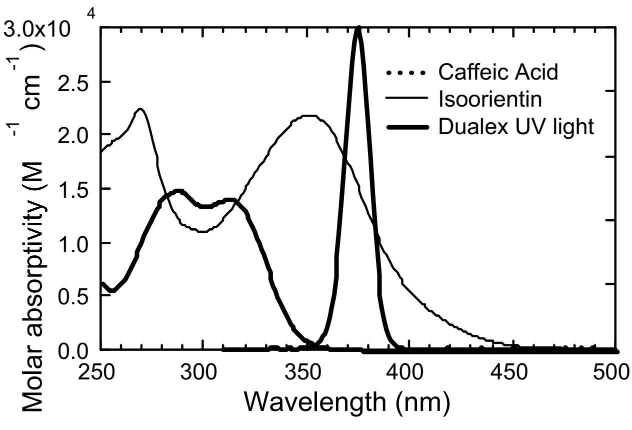

Figure 1. Spectra of the main wheat-leaf absorbers compared to the UV light source of the Dualex® leaf-clip.

Materials and methods

Field experiments

Experiments were conducted at four different locations in France, during three growing seasons (2001-2003), and on four cultivars: winter wheat (*Triticum aestivum*) cv. Isengrain, Récital and CapHorn, and durum wheat (*Triticum durum*) cv. Joyau. Details of these experiments can be found in Cartelat *et al.* (2005). The timing of phenological events is given according to the Zadoks' scale (Zadoks *et al.*, 1974). Nitrogen was applied in three or four top dressings varying from 40 to 100 kg ha^{-1} (cf. Figure 4C). Crops were fully protected against weeds and pests.

Optical measurements

The Dualex® (FORCE-A, France) technology, involving the use of ChlF screening by UV-absorbing compounds of the epidermis, is described by Goulas *et al.* (2003; 2004). A commercial leaf-clip, SPAD-502 (Minolta, Japan), was used for Chl estimation. Where indicated, SPAD values were transformed into chlorophyll units ($\mu g\ cm^{-2}$) using a calibration curve (Cartelat *et al.*, 2005). Leaf-clip measurements were made on the middle of the uppermost fully expanded leaves. The Chl data presented are means of measurements made from the adaxial side of 30 leaves, and the Phen data presented are means of the sum of measurements of the adaxial and the abaxial side of 30 leaves, except for data in Figure 3, where each point represents 20 to 25 leaves. The percentage standard deviation was around 10% for all data.

Nitrogen analyses

Total-N concentration in shoots, on a dry matter basis, was determined using the Dumas method. (NA 1500 analyser, Fisons Instruments). For leaf-N quantification, segments of leaves where optical measurements had been made, were freeze-dried and finely ground and a 2 to 3 mg aliquot of the powder combusted in an elemental analyser (Model NA1500, Carlo Erba). Nitrogen content was determined by gas chromatography. Leaf N content is expressed on a dry weight basis, in mg g^{-1}. Grain protein content (GPC) was measured using a near-infrared instrument InfraAlyser (Model 400, Bran & Luebbe, Australia), calibrated with the Kjeldahl method of protein determination.

Statistical analysis

Curve fitting and statistical analysis were performed using Igor Pro 4.0 software (WaveMetrics, USA).

Results and discussion

Indicators of N content

The N content of the uppermost fully expanded leaf provided a good representative of overall crop N status (Figure 2A). Therefore, Chl and Phen contents of this leaf may be used as indicators of crop N status (Figure 2B). Thanks to the opposite dependences of Chl and Phen on N (Figure 2B), the combined indicator, Chl/Phen, was highly dependent on leaf N content (Figure 2C).

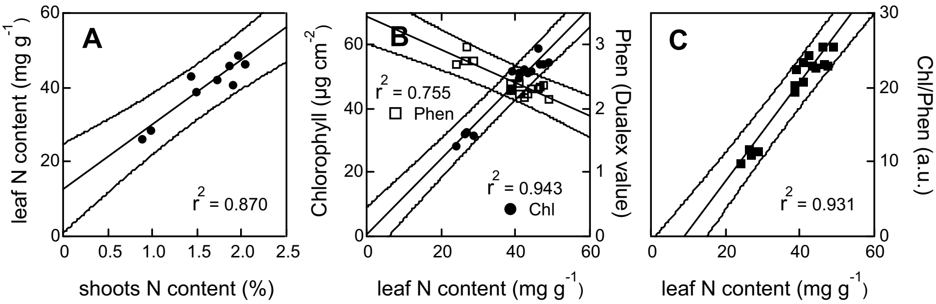

Figure 2. Relationship between (A) the uppermost leaf and the whole shoot N content, (B) leaf Chl and leaf Phen and leaf N content, and (C) leaf Chl/Phen ratio and leaf N content - with linear fits and 95 % confidence limits shown. Cv. Isengrain and Récital, growth stages from end of stem elongation (Z37) to flowering (Z65).

Predicting grain quality from optical indicators in the field

Although low leaf N contents can be obtained experimentally, in real agricultural field situations N nutrition is usually medium to high. In such situations Chl changes are small and limit its predictive value (Hoel, 2002; Lopez-Bellido *et al.*, 2004). For example, in all fields referred to in Figure 3, residual mineral N in soil at the end of winter was high, 70 kg N ha^{-1}. Consequently, Chl was already high for the unfertilised crop, and increased only slightly with additional dressings of N up to 250 kg ha^{-1} (Figure 3A). Phen responded more than Chl to increased N rate (Figure 3B). These differences were also evident in the relationship between optical indicators and harvested grain quality (Figure 3D & 3E). The high GPC of the durum samples could not be predicted from leaf Chl content at growth stage Z37 (flag leaf just visible) since the relationship between Chl and GPC levelled out above a GPC of 12% (Figure 3D), whereas it was predictable with Phen as shown in Figure 3E. Hence, the Chl/Phen ratio correlated better with GPC than Chl alone, for both soft and durum wheat analysed separately and together (Table 1).

Table 1. Correlation analysis of different optical indicators (Chl, Phen, Chl/Phen) with the grain protein content (GPC) for soft and durum wheat. Pearson's correlation coefficients are presented. n = number of measurements.

GPC	n	Chl	Phen	Chl/Phen
Durum wheat	5	0.717ns	-0.975**	0.937*
Soft wheat	12	0.796**	-0.932***	0.927***
Soft and durum wheat	17	0.657**	-0.842***	0.844***

Level of probability: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; ns = not significant

Sensitivity and variability of optical indicators

The absolute difference in Chl between N-deficient (0 N added) and N-sufficient (260 kg ha^{-1} added in total) wheat constantly increased during the growth season (Figure 4A). The absolute difference in Phen, except at the end of winter, did not change much (Figure 4B). Still, if we express

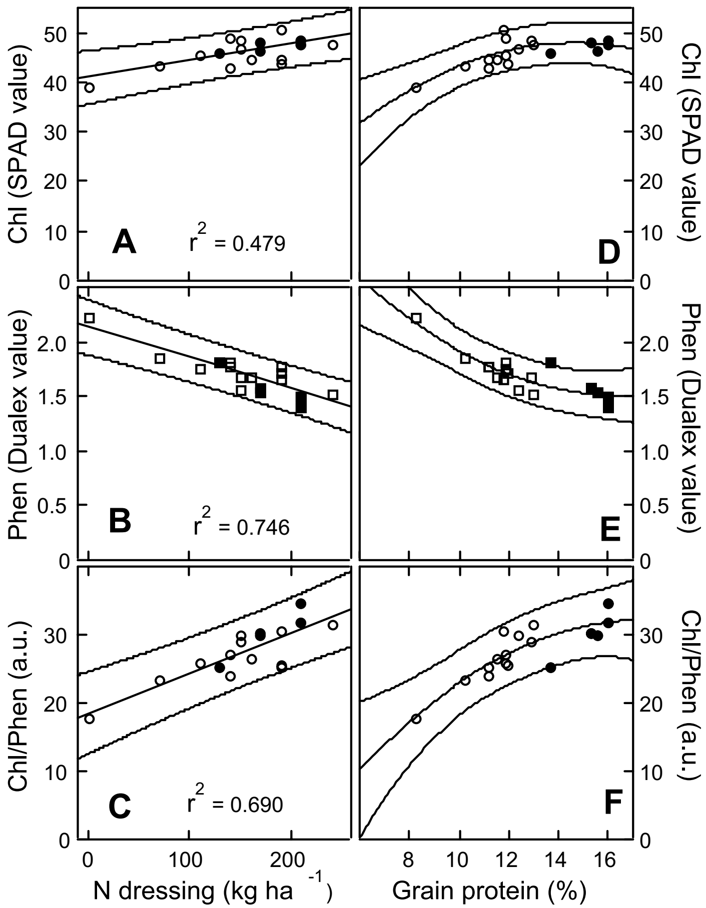

Figure 3. Optical indicators response to N top dressing (A, B, C) and as predictors of GPC (D, E, F) at the Z37 growth stage. Open and closed symbols are for winter wheat (Isengrain, CapHorn) and durum wheat (Joyau) cultivars, respectively. The full lines are linear fits (for N dressing) or quadratic polynomial fits (for GPC). Dashed lines are prediction bands for the 95 % confidence level. Each point corresponds to one field.

the sensitivity of optical indicators as the normalised difference between the value measured on N-sufficient (N_{max}) and N-deficient (N_0) plants ((N_{max} - N_0)/ N_{max}), then it can be seen that the sensitivity increased during the growing season for both Chl and Phen (Figure 4C). The difference is negative for Phen because its content decreases with N fertilisation. The largest increase in sensitivity is from the end of tillering (Z30) until the second node is detectable (Z32-growth stage), with a small decrease at the end of elongation. Chl was more sensitive to N-deficiency than Phen in this example, because the residual N was very low, less than 20 kg ha^{-1}, inducing a low Chl content in N_0 plants throughout the season. Still, the combined indicator, Chl/Phen, increased the sensitivity substantially, by 30 % in most cases (from 0.4 to 0.6) (Figure 4C).
The presently accepted practice is to measure Chl in the middle of the leaf (e.g. Hoel, 2002). This is compulsory because there is a gradient of Chl content of about 20% along the leaf in monocots due to the difference in cell age (Meyer *et al.*, 2003; Cartelat *et al.*, 2005). For the same reason,

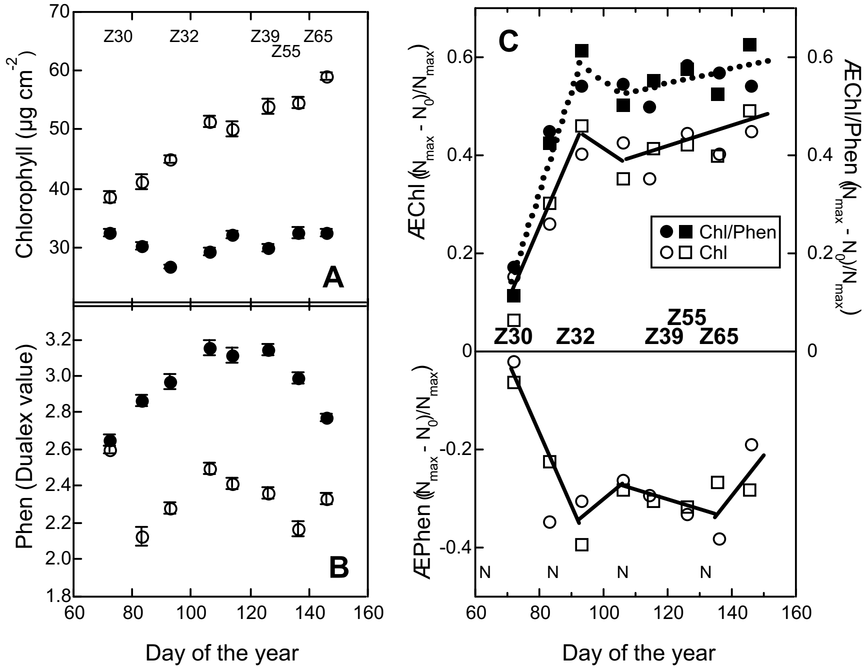

Figure 4. The sensitivity of optical indicators during the growth season. Phen (A) and Chl (B) leaf content (means and standard errors for 30 leaves) are presented for N-deficient (closed symbols) and N-sufficient (open symbols) winter wheat cv. Isengrain. In C, the calculated sensitivities are presented for two cultivars, Isengrain (circles) and Récital (squares), where N indicates the dates when nitrogen was applied (at 60, 80, 80 and 40 kg/ha, respectively).

wheat leaves show a gradient in Phen content, in the same direction as Chl, increasing from ligula towards the tip (Cartelat *et al.*, 2005). This physiological feature confers an increased robustness on the Chl/Phen ratio that varies less than 10 % along the leaf, and gives a better separation of leaves having small differences in mean Chl content (data not shown).

The protein competition model of polyphenolic allocation

Although mainly flavonoid absorbance was measured by the Dualex®, the data presented reflect the behaviour of total Phen, confirming the PCM and associated hypotheses on wheat (cf. also Cartelat *et al.*, 2005). This implies that Phen can be used as an additional optical indicator of N status provided it is measured in the uppermost leaves. The latter are exposed to full sunlight, so their photosynthesis insures that carbon availability is not limiting, and therefore the dependence on N is evident.

Usefulness of the Chl/Phen ratio as indicator of N status

Optical methods for Chl and Phen yield data on a leaf surface basis, and likewise photosynthetic rate is expressed on a surface area basis. In contrast, ecophysiological and agronomic measurements are usually expressed in mass-based units, because it is the fate of assimilated carbon and N that is being investigated. The link between the two types of measurements is the

LMA, a variable difficult to obtain, and not appropriate for precision agriculture because it is destructive. The use of the ratio of two optical surface-based values should alleviate this dependence on LMA. As both Chl and Phen can be measured remotely using active sensors (Ounis *et al.*, 2001), the implementation of the Chl/Phen ratio in future real-time DSS may be possible. Because of the importance of grain protein content for soft wheat, and even more for durum wheat, farmers tend to use large quantities of N fertilisers. This decreases the predictive value of simple Chl measurements because this indicator tends to saturate at high leaf N contents (Hoel, 2002; Lopez-Bellido *et al.*, 2004). The main advantage of the Chl/Phen indicator is its higher predictive value for grain quality including for durum wheat (cf. Figure 3 and Table 1). It will permit quality assurance and field selection for type of grain quality. A second advantage is that the sensitivity of the combined indicator is higher than that of Chl alone as Chl varies directly and Phen inversely with N supply. The Chl/Phen ratio has an additional advantage of alleviating, at least partially, the longitudinal heterogeneity in leaf Chl and Phen content (Cartelat *et al.*, 2005). The effect of this heterogeneity on optical signals measured at the canopy level has not been studied in depth yet.

Conclusions

The combined Chl and Phen measurement can improve the precision of nondestructive optical indicators at the leaf level, for N management during the growth season, and for predicting GPC. The Chl/Phen ratio has a good sensitivity to wheat N status and is better adapted for remote sensing, therefore it has potential for real-time site-specific N management.

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