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Use of blue–green and chlorophyll fluorescence measurements for differentiation between nitrogen deficiency and pathogen infection in winter wheat

Kathrin Bürling, Mauricio Hunsche*, Georg Noga

University of Bonn, Institute of Crop Science and Resource Conservation (INRES) - Horticultural Science, Auf dem Hügel 6, D-53121 Bonn, Germany

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ABSTRACT

In recent years, several sensor-based approaches have been established to early detect single plant stresses, but the challenge of discriminating between simultaneously occurring stressors still remains. Earlier studies on wheat plants strongly affected by pathogens and nitrogen deficiency indicated that chlorophyll fluorescence might be suited to distinguish between the two stressors. Nevertheless, there is lack of information on the pre-symptomatic detection of synchronized occurrence of slight N-deficiency and the early stages of pathogen infection. The usefulness of the blue, green, and yellow fluorescence signals in this context has not yet been explored. We hypothesized that differentiation between wheat plants' physiological reaction due to N-deficiency and leaf rust (*Puccinia triticina*) as well as N-deficiency and powdery mildew (*Blumeria graminis* f. sp. *tritici*) might be accomplished by means of UV laser-induced fluorescence spectral measurements between 370 and 620 nm in addition to chlorophyll fluorescence (640–800 nm). Plants were provided with either a normal or a modified Hoagland nutrient solution in order to induce a slight N deficit. Pathogen inoculation was carried out on the second fully developed leaf. Four experimental groups were evaluated: (a) N-full-supply [N+]; (b) N-deficiency [N−]; (c) N-full-supply + pathogen [N+/LR] or [N+/PM]; (d) N-deficiency + pathogen [N−/LR] or [N−/PM]. The results revealed that, in addition to the amplitude ratio of R/FR fluorescence, B/G fluorescence also facilitated reliable and robust discrimination among the four experimental groups. The discrimination among the experimental groups was accomplished as early as one and two days after inoculation for powdery mildew and leaf rust infection, respectively. During the 3 days evaluation period, the differences among the treatment groups became more evident. Moreover, several other amplitude ratios and half-bandwidth ratios proved to be suited to early detect fungal infection, irrespective of the nitrogen status of the plant.

Introduction

The incidence of disease and deficiency of nutrients representing biotic and abiotic stresses, respectively, are the limiting factors for crop production worldwide. As estimated, the potential of yield loss of wheat due to fungal pathogens might amount to 15% under specific conditions (Oerke and Dehne, 2004). During wheat's life cycle, wheat plants are often infected by the biotrophic fungi *Puccinia triticina* and *Blumeria graminis* f. sp. *tritici*, causing leaf rust and powdery mildew, respectively. On the other hand, nitrogen is a key element in plant nutrition (Marschner, 2005), and its adequate supply is the most important nutritional process a farmer can manage in cultivated crops (McMurtrey et al., 1994).

In recent years, with advanced technology, sensing of stress-induced alterations of metabolism and crop physiology have become increasingly of interest to detect modifications at early stages before extensive plant damage occurs. For this purpose, several non-destructive approaches, e.g. fluorescence, reflectance, and thermal-imaging measurements, have been evaluated and adopted for the fast and early detection of individual stresses, such as diseases (e.g. Bodria et al., 2002; Bravo et al., 2003; Franke and Menz, 2003; Lindenthal et al., 2005; Kuckenberg et al., 2009b) and mineral deficiency, and especially the nitrogen status of plants (e.g. Bredemeier et al., 2003; Schächtl et al., 2005; Subhash and Mohanan, 1994; Tartachnyk and Rademacher, 2003; Buschmann, 2007). In addition to promising results, the specificity of the measuring system and the particularities of the experiments must be considered when evaluating or comparing suitable techniques. As recently shown, the chlorophyll fluorescence imaging techniques appear to be more sensitive than thermal imaging for early

Abbreviations: A, absorbance; AOTF, acusto-optic tunable filter; B, blue; *B. graminis*, *Blumeria graminis*; Chl*t*, total chlorophyll; dai, day after inoculation; DMSO, dimethyl sulfoxide; F, fluorescence; FR, far-red; FW, fresh weight; G, green; hai, hours after inoculation; *hbw*, half-bandwidth; LR, leaf rust; N−, N-deficiency; N+, N full-supply; *P. triticina*, *Puccinia triticina*; PAR, photosynthetic active radiation; PM, powdery mildew; PMT, photomultiplier; R, red; RD, resistance degree; SVM, Support Vector Machines; UV, ultra-violet; UV–VIS, ultra-violet–visible.

* Corresponding author. Tel.: +49 228 73 6540; fax: +49 228 73 5764.
E-mail address: MHunsche@uni-bonn.de (M. Hunsche).

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detection of pathogen infection and nutrient deficiency (Chaerle et al., 2007a). Both nitrogen deficiency and pathogen infection are accompanied by a decrease in chlorophyll content (Tartachnyk and Rademacher, 2003).

In general, reliable detection of stress can be achieved when evaluating biotic and abiotic stresses as single factors. However, it is not unusual that several stresses influence the plant physiology simultaneously. Despite considerable advances, reliable discrimination between biotic and abiotic stresses using non-destructive techniques remains a challenge. Tartachnyk et al. (2006). showed that discrimination between strong N-deficiency and pathogen infection at advanced stages can be accomplished on the basis of fluorescence peak ratio F690/F730. However, as shown for a cross-validation analysis of chlorophyll fluorescence, diseased leaves could be misidentified as N-deficiency and vice versa, whereas the classification was improved when the standard deviation of the mean was also considered as a parameter for discrimination (Kuckenberg et al., 2009a). Unfortunately, these conclusions were based on pathogen infection and N-deficiency evaluated on different leaves, whereas research studying both stressors concomitantly on the same plants is scarce.

When exposed to stresses, specific pigments and other molecules might be synthesized, accumulated or degraded, having an indirect or direct influence on the fluorescence signature. In general, nitrogen deficiency leads to less chlorophyll in the tissues (Ciompi et al., 1996). Furthermore, nitrate availability influences not only the chlorophyll concentration and therefore chlorophyll fluorescence, but also phenol and lignin production, which are reduced in wheat shoots by high nitrate levels (Brown et al., 1984). When infected with fungal pathogens, plants might accumulate specific substances, such as salicylic acid and phenylpropanoid compounds (e.g. cinnamic acid, stilbens, coumarins and flavonoids) as the most important substances in plant disease resistance (Chaerle et al., 2007b; Lenk et al., 2007). Accordingly, the fluorescence in the blue–green spectral range might yield promising results, since it has been proven to be very sensitive to single stress events reflecting, among others, accumulation of secondary metabolites (Lichtenthaler and Miehé, 1997; Cerovic et al., 1999; Buschmann et al., 2009). However, the suitability of the fluorescence outcome in the blue, green, and yellow spectral range for discriminating stressors is not yet proven. Therefore, we hypothesized that differentiation between wheat plants' physiological reactions due to N-deficiency and leaf rust (*Puccinia triticina*) as well as N-deficiency and powdery mildew (*Blumeria graminis* f. sp. *tritici*) might be accomplished by means of UV laser-induced fluorescence spectral measurements in the blue, green and yellow range (370–620 nm) in addition to the chlorophyll fluorescence (640–800 nm). We focused on a slight N-deficiency and the early stages of pathogen infection, based on the need of sensors to detect pre-symptomatic stress signals. Of primary interest was the basic suitability of combined spectral information by evaluation of several fluorescence ratios for consideration in future field experiments requiring more complex and developed detection systems.

Materials and methods

Plant material

Experiments were conducted in a controlled-environment cabinet simulating a 14-h photoperiod with 200 $\mu M\,m^{-2}\,s^{-1}$ photosynthetic active radiation (PAR; Philips PL-L 36W fluorescent lamps, Hamburg, Germany), day/night temperature of $20/15 \pm 2\,^{\circ}C$ and relative humidity of $75/80 \pm 10\%$. Winter wheat (*Triticum aestivum* L. emend. Fiori. et Paol.) seeds of the leaf rust (LR) and powdery mildew (PM) susceptible cultivar Ritmo were sown in individual pots (5 seeds per pot) filled with perlite. According to the descriptive variety list of the German Federal Plant Variety Office (2008), Ritmo is classified with a resistance degree (RD) of 8 for leaf rust and RD = 5 for powdery mildew, in a classification range from one (resistant) to nine (susceptible). Inoculation of single leaves with either leaf rust or powdery mildew was carried out on the second fully developed leaf, twenty days after sowing. Experiments with combined nitrogen supply and leaf rust or powdery mildew inoculation were conducted separately and repeated at least twice. Accordingly, the experimental setup was as follows: (a) N-full-supply [N+]; (b) N-deficiency [N−]; (c) N-full-supply + pathogen [N+/LR] or [N+/PM]; (d) N-deficiency + pathogen [N−/LR] or [N−/PM]. In each experiment, the number of replications was $n = 12$ for the nitrogen treatments, and $n = 16$ for the nitrogen + pathogen treatments. Pathogen inoculation was performed on two plants per pot.

Fertilization and chlorophyll determination

Emerging plants were provided with either a standard or a modified Hoagland nutrient solution; the first contained all mineral nutrients for optimal plant growth and development, and the second was adjusted to induce nitrogen deficiency. Several pre-experiments with defined amounts of nitrogen were conducted in order to determine the appropriate N concentration to induce a slight N deficit that is not evident by visual observation. The full nitrogen supply solution (N+) contained 236.16 $g\,L^{-1}$ $Ca(NO_3)_2{\cdot}4H_2O$, 57.54 $g\,L^{-1}$ $NH_4H_2PO_4$, and 67.74 $g\,L^{-1}$ KNO_3 whereas the N-deficiency solution contained 68.05 $g\,L^{-1}$ KH_2PO_4, 74.55 $g\,L^{-1}$ KCl, 126.12 $g\,L^{-1}$ $Ca(NO_3)_2{\cdot}4H_2O$, and 13.2 $g\,L^{-1}$ $(NH_4)_2SO_4$. Consequently, the deficiency solution (N−) contained 40% of the N-amount of the standard solution. The content of micronutrients was similar in both fertilization solutions.

Leaves at the same developmental stage as those used in the main experiments were sampled and the nitrogen status was evaluated non-destructively with a chlorophyll meter (SPAD 502, Konika Minolta, Langenhage, Germany) on the adaxial sides of the leaves by measuring red (~650 nm) and infra-red (~940 nm) light transmission. From the same leaves, chlorophyll content was extracted from 1 cm^2 leaf pieces with dimethyl sulfoxide (DMSO) and analytically determined as described elsewhere (Blanke, 1992). The absorbance of extracts was evaluated at 665 nm (A665) and 647 nm (A647) with a UV–VIS spectrophotometer (Perkin-Elmer, Lambda 5, Massachusetts, USA). Total chlorophyll (Chl*t*) concentration was calculated on fresh-weight basis according to the equation: $Chlt = 17.9 \times A647 + 8.08 \times A665$.

Pathogen inoculation

Inoculation of Puccinia triticina

Inoculation was carried out with a non-specific mixture of *Puccinia triticina* spores produced on wheat without known resistance genes (INRES - Phytomedicine, University of Bonn). Before each experiment, fresh *P. triticina* spores were suspended in a solution of distilled water + Tween 20 (0.01%, w/v; Merck-Schuchardt, Hohenbrunn, Germany). The spore concentration was estimated microscopically with a Fuchs-Rosenthal counting chamber and adjusted to 1×10^4 spores mL^{-1}. On each leaf, the middle of the leaf length was marked on the adaxial side with a felt tip pen, and seven 6-μL droplets of spore suspension were applied in a row on one leaf half (Fig. 3). Prior to the application, leaves were fixed horizontally on a sample holder to prevent droplet run-off. During the inoculation period (22 h), plants were maintained in the climate chamber at almost water vapor saturated atmosphere ensured by a plastic cover. Thereafter, the plastic cover was removed and the leaves were released from their horizontal fixation. Plants of the groups without pathogen inoculation were handled similarly but

treated with water droplets + Tween 20 (0.01%, w/v). Fluorescence measurements were performed on the central of the seven droplet application sites. The development of disease spots was evaluated visually over the experimental period *in situ* and on digital photographs taken in parallel to the fluorescence readings.

Inoculation of Blumeria graminis

Similar to the methods described for the inoculation of leaf rust, the target leaves were selected and the middle of leaf length was marked with a felt tip pen. Marked leaves were horizontally fixed before inoculation with conidia of a non-specific mixture of *Blumeria graminis* f. sp. *tritici* produced on wheat without known resistance genes (INRES - Phytomedicine, University of Bonn). Stock plants inoculated with the pathogen ensured the supply of fresh conidia when needed. For inoculation of experimental plants, conidia were carefully removed from the stock plants with a fine brush and directly applied on the leaf surface of the target plants. Application site (3 × 5 mm) was located at the leaf length middle in the centre of a leaf half. Twenty-two hours after inoculation (hai), visible conidia were removed by gently blowing and brushing over leaf surface. Leaves of the plant groups N+ and N− were handled in a similar way without conidia.

Fluorescence measurements

Fluorescence measurements were carried out using a compact fiber-optic fluorescence spectrometer with nanosecond time resolution and employing the boxcar technique (IOM GmbH, Berlin, Germany). A pulsed N_2 laser (MNL100, LTB Lasertechnik Berlin GmbH, Berlin, Germany) with an emission wavelength of 337 nm and a repetition rate of 20 Hz served as the excitation source. The fiber-optic probe for detection of fluorescence signals consisted of a central excitation fiber and six surrounding emission fibers, each with a 200-μm diameter. The pulse energy at the probe exit was adjusted to be in the range of 1.5–3.0 μJ with a pulse length of approximately 2.5 ns resulting in a density of 7.5–15 × 10^{15} photons per cm^2 and pulse. Fluorescence was recorded with an acousto-optic tunable filter (AOTF) monochromator, which enables a minimal step width of 1 nm. A photomultiplier (PMT, H5783-01, Hamamatsu, Hamamatsu City, Japan) was used as detector. The sensitivity of the PMT was adjusted to optimize the signal intensity during the spectral measurements. Time resolution was accomplished using a gated integrator with a 2-ns half-width; positioning of the gate allowed an accuracy of 0.1 ns.

Detection of fluorescence spectra was carried out on leaves fixed horizontally on a plate with integrated sample holder. The fiber-optic probe was positioned at a 90° angle to the leaf. By employing a laser-based rangefinder (OptoNCDT 1300; Micro-Epsilon Messtechnik GmbH & Co. KG, Ortenburg, Germany) fixed beside the probe, the distance between leaf and probe surface was adjusted to 3.95 mm at the point of measurement. The standard distance enabled fluorescence intensities in a narrow data range, providing a minimum of signal intensity and avoiding signal saturation. Spectra were measured at 21–23 °C under ambient light conditions (about 18 μM m^{-2} s^{-1} PAR) at two to four days after pathogen inoculation (dai) for leaf rust experiments, and one to three dai for powdery mildew experiments. Prior to fluorescence measurements, plants were adapted for 0.5 h to room conditions. For optimization of fluorescence signals, equipment settings were adjusted as follows. Spectral analysis was accomplished at a wavelength interval of 2 nm between 370 and 800 nm with a gate position at 5 ns (in the temporal signal maximum). Measurements were done with a pulse count of 32, which is the number of laser pulses averaged for each single data point. The PMT sensitivity was set to 600 Volt. Fluorescence peaks were determined at 451 nm (blue, B), 522 nm (green, G), 689 nm (red, R), and 737 nm (far-red, FR).

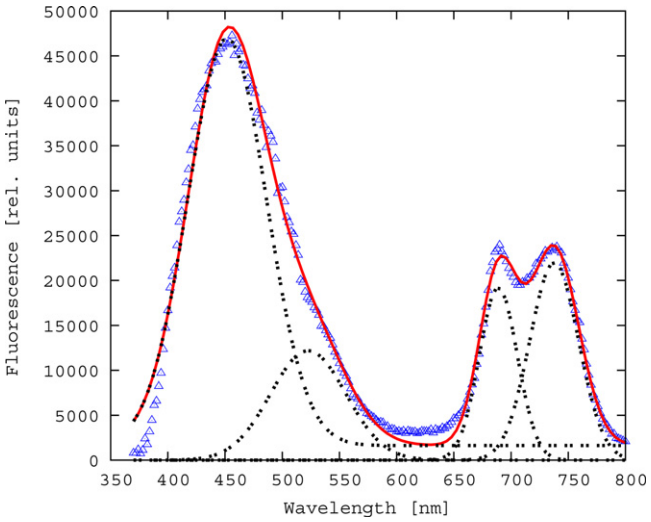

Fig. 1. Example of fluorescence spectra (370–800 nm) recorded from a healthy wheat leaf of the N-full-supply treatment group with a fluorescence spectrometer with nanosecond time resolution using a pulsed nitrogen laser (337 nm) as excitation source. The triangles indicate the measured fluorescence emission spectrum, the dotted lines display the individual Gaussian spectral components of the fitted spectrum, and the solid line shows the fitted spectrum.

Data processing and statistics

The measured laser-induced fluorescence spectra were processed by Gaussian curve fitting using the freeware Gnuplot (version 4.2 patchlevel 4, http://www.gnuplot.info, Geeknet Inc., Mountain View, CA, USA), as indicated in Fig. 1. Position, amplitudes as well as half-bandwidths (*hbw*) of peaks were determined to calculate the ratios between amplitudes, half-bandwidths, and amplitudes-to-half-bandwidths (*) for individual peaks. The processed experimental data were subjected to statistical analysis using the SPSS package (SPSS Inc., Chicago, USA) version 18.0. The relation between SPAD and chlorophyll content was established with a linear regression. For each day and evaluation group, the means were compared by ANOVA ($p \leq 0.05$) and means separated with the Duncan's multiple range test. Graphs (mean ± SD) were drawn with SigmaPlot 8.02 (Systat Software Inc., Richmond, CA, USA).

Results

Validation of N-deficiency

The chlorophyll content of plant treatments with N+ and N− was evaluated to show that the N− treatment group was nitrogen deficient even if visual symptoms were not evident. On the basis of SPAD-values and chlorophyll extraction, a linear function expressed as Chl [μg g^{-1} FW] = 53.34 × SPAD − 248.024 (r^2 = 0.93) was established (Fig. 2). However, the usefulness of the proposed linear function might be limited to our experimental conditions (hydroponic cultivation of the wheat cultivar Ritmo receiving either full N-supply or 40% of N), whereas for a wide range of chlorophyll concentrations and SPAD values, non-linear curves seems to be more appropriate (Uddling et al., 2008). On average, leaf chlorophyll concentrations of N+ plants were 2311 μg g^{-1} FW, whereas the leaves of the N− treatment group had a mean of 1698 μg g^{-1} FW. Aside from this significant difference, the visual assessment of N-deficiency leaves revealed no distinct stress symptoms (Fig. 3A).

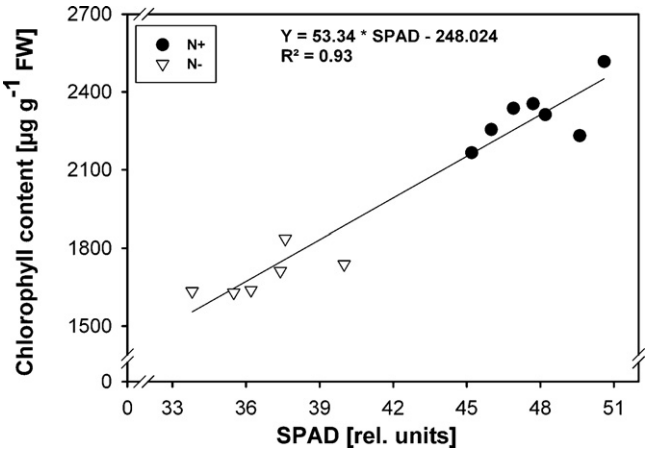

Fig. 2. Correlation between SPAD values and chlorophyll content of wheat leaves as affected by two levels of nitrogen supply ($n \geq 6$).

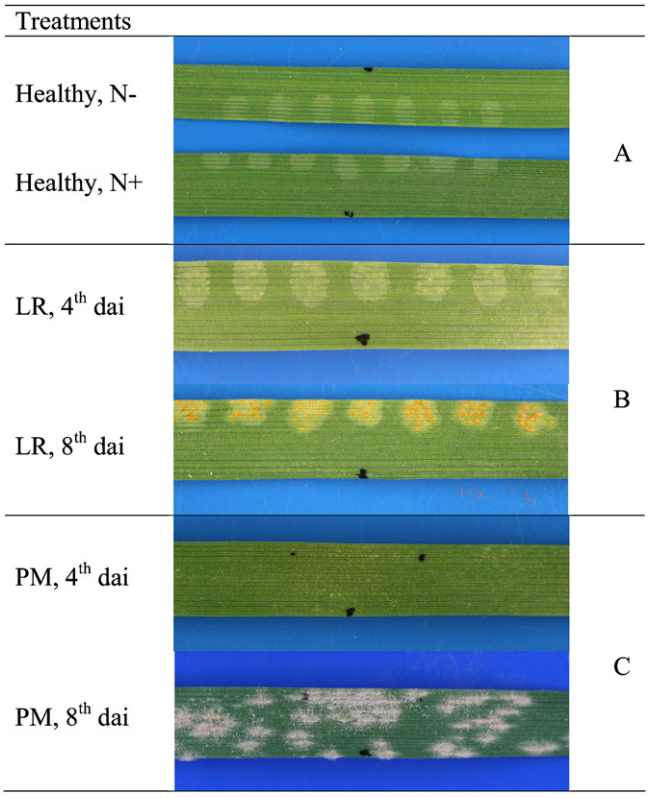

Fig. 3. Digital photographs of wheat leaves affected by abiotic or biotic stress factors: (A) influence of nitrogen fertilization, N-full-supply (N+) and N-deficiency (N−); (B) leaves infected by leaf rust, four and eight days after inoculation (dai); (C) infection of powdery mildew at four and eight days after inoculation.

Combined nitrogen deficiency and leaf rust infection

Visual evaluations of leaf rust development indicated small and loom chlorotic spots 4 dai (days after inoculation) on the adaxial leaf lamina in both nitrogen full supply (N+) and nitrogen deficient (N−) leaves (Fig. 3B). Two days later (6 dai), small red-brown pustules appeared on the leaf surface and became larger and more distinct in the following days. After 8 dai, disease symptoms were evident (Fig. 3B).

The spectrally resolved fluorescence measurements and the identification of peak maxima at 451 nm (B), 522 nm (G), 689 nm (R), and 737 nm (FR) (Fig. 1) allowed us to calculate six amplitude and six half-bandwidths ratios (B/G, B/R, B/FR, G/R, G/FR, R/FR) as well as four amplitudes-to-half-bandwidth ratios (B, G, R, FR). However, not all ratios are appropriate to detect N-deficiency and pathogen infection on the same leaves. We therefore focused on the most promising ones. As shown in Table 1, several of the examined fluorescence ratios facilitated reliable discrimination between healthy and inoculated leaves from 2 to 4 dai, irrespective of nitrogen fertilization. Amplitude ratios of B/R, B/FR, G/R and G/FR were significantly higher in inoculated than in non-inoculated leaves. Two dai, values for B/R were 2.98 and 3.01 for N+ and N−, and 3.53 and 3.37 for N+/LR and N−/LR, respectively (Table 1). In a similar trend, values of the G/R ratio were 0.77 and 0.80 for N+ and N− and 0.96 for both nitrogen variants inoculated with the leaf rust pathogen. In addition, the pathogen inoculation reduced the B/G and increased the G/FR_*hbw* and R/FR_*hbw* ratios (Table 1). Of all the evaluated amplitude-to-half-bandwidth ratios, a clear difference between the treatment groups was observed for the FR* peak, showing values of 923 (N+), 920 (N−), 796 (N+/LR) and 788 (N−/LR) at two days after inoculation (Table 1). The difference between healthy and inoculated leaves became slightly greater during 3 days of measurements, as indicated by values of B/R (Table 1).

To distinguish among the four experimental groups, two fluorescence ratios were considered as promising parameters. On all the three measuring days (2–4 dai) the amplitude ratio B/G was significantly different among the four groups (Fig. 4A). At the 2nd dai, leaves of N+ plants had the highest values (3.89), followed by N− (3.75), N+/LR (3.68), and N−/LR (3.50) (Fig. 4A). In all treatments, values decreased over time due to leaf aging and at the 4th dai, one day before first visible symptoms appeared, ratios

Table 1
Influence of nitrogen supply (N+, full-supply; N−, 40% of full-supply) and leaf rust (LR) inoculation on selected fluorescence ratios, determined from two to four days after inoculation (dai).

Fluorescence ratio	Experimental group	2 dai	3 dai	4 dai
B/R	N+	2.98 a	2.55 a	2.24 a
	N+/LR	3.53 b	3.09 b	2.91 b
	N−	3.01 a	2.55 a	2.29 a
	N−/LR	3.37 b	3.04 b	3.00 b
B/FR	N+	2.81 a	2.34 a	2.06 a
	N+/LR	3.50 b	3.22 b	3.38 b
	N−	2.90 a	2.44 a	2.20 a
	N−/LR	3.49 b	3.37 b	3.80 b
G/R	N+	0.77 a	0.68 a	0.62 a
	N+/LR	0.96 b	0.93 b	1.00 b
	N−	0.80 a	0.71 a	0.65 a
	N−/LR	0.96 b	0.97 b	1.09 b
G/FR	N+	0.72 a	0.62 a	0.57 a
	N+/LR	0.95 b	0.97 b	1.17 b
	N−	0.77 a	0.67 a	0.63 a
	N−/LR	1.00 b	1.07 b	1.39 b
B/G_*hbw*	N+	1.07 b	1.06 b	1.05 b
	N+/LR	1.06 a	1.03 a	1.01 a
	N−	1.08 b	1.06 b	1.06 b
	N−/LR	1.06 a	1.03 a	1.01 a
G/FR_*hbw*	N+	1.52 a	1.52 a	1.53 a
	N+/LR	1.55 b	1.58 b	1.59 b
	N−	1.51 a	1.52 a	1.52 a
	N−/LR	1.54 b	1.56 b	1.58 b
R/FR_*hbw*	N+	0.79 a	0.78 a	0.78 a
	N+/LR	0.80 b	0.79 b	0.79 b
	N−	0.79 a	0.79 a	0.78 a
	N−/LR	0.79 b	0.79 b	0.79 b
FR*	N+	923 b	956 b	975 b
	N+/LR	796 a	784 a	739 a
	N−	920 b	933 b	941 b
	N−/LR	788 a	753 a	694 a

Means of the fluorescence parameters for each evaluation day followed by the same letter do not differ significantly according to Duncan's multiple range test ($p \leq 0.05$; $n = 12$ for N+ and N−; $n = 16$ for N+/LR and N−/LR). *hbw* = half-bandwidth, * = amplitude-to-half-bandwidth ratio.

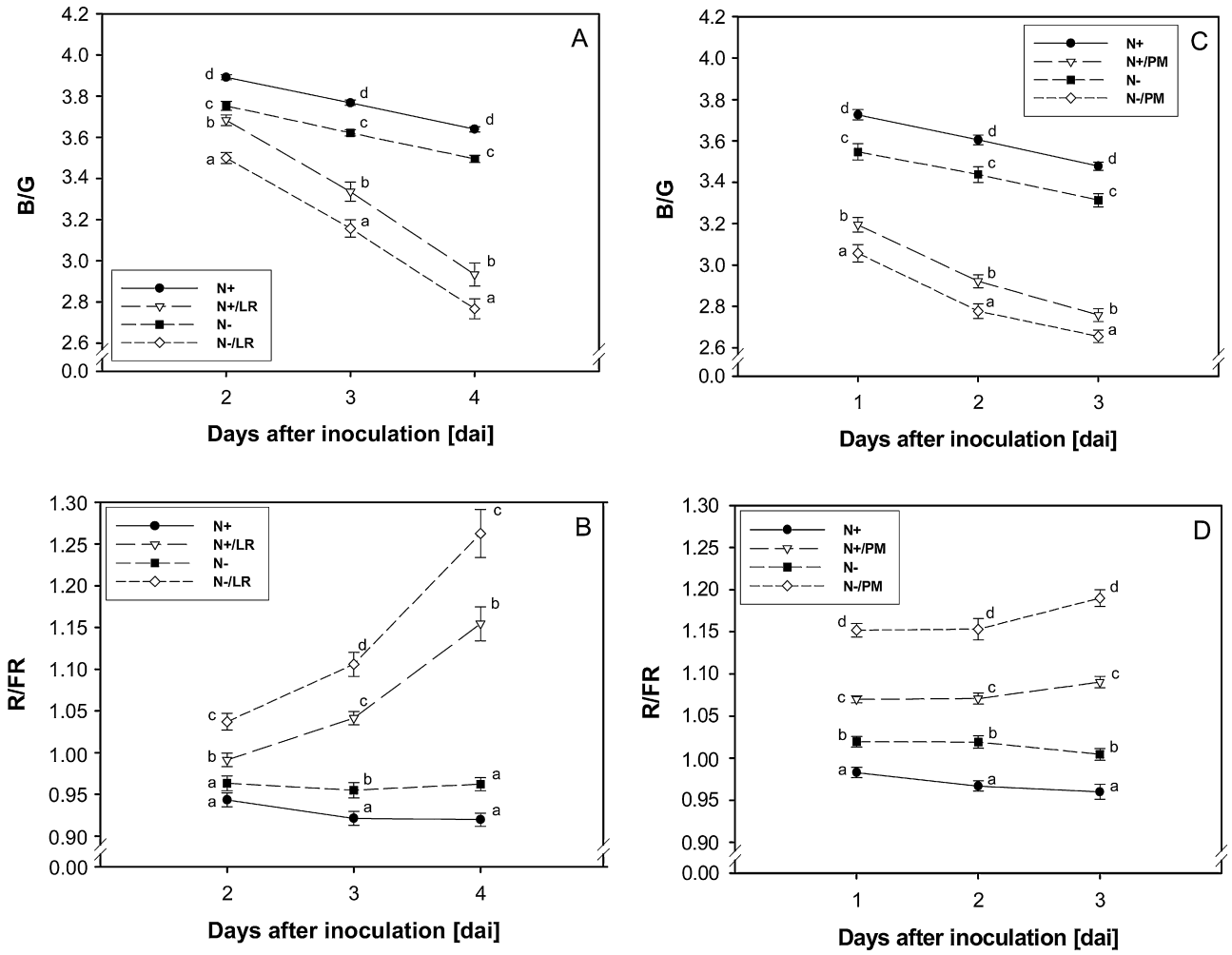

Fig. 4. Influence of nitrogen supply (N+, full-supply; N−, 40% of full-supply) and disease development on the ratios of fluorescence amplitudes B/G and R/FR. (A and B) Leaf rust (LR) infection measured from two to four days after inoculation (left); (C and D) powdery mildew (PM) infection, measured from one to three days after inoculation (right). Means (±SD) of the experimental groups (within each evaluation day) followed by the same letter do not differ statistically according to Duncan's multiple range test ($p \leq 0.05$; $n = 12$ for N+ and N−; $n = 16$ for the other treatment groups).

reached 3.64 for N+, 3.50 for N−, 2.93 for N+/LR and 2.77 for N−/LR (Fig. 4A). Consequently, the difference between N+ and N− as well as between N+/LR and N−/LR remained the same, but the difference between inoculated and non-inoculated leaves became greater. Measurements of the chlorophyll fluorescence in the R and FR peaks indicated slightly higher amplitude ratios R/FR in inoculated (N+, 0.94; N−, 0.96) than in non-inoculated (N+/LR, 0.99; N−/LR, 1.04) (Fig. 4B) leaves. Values for N+ and N− remained almost constant, while the inoculated leaves showed a strong increase (Fig. 4B).

Combined nitrogen deficiency and powdery mildew infection

Visual evaluations of powdery mildew development first revealed small patches of whitish mycelium on the leaf surface at four days after inoculation (Fig. 3C). During the following days the patches increased in size, and new mycelia were formed. As with leaf rust, a large number of amplitude, half-bandwidth, and amplitude-to-half-bandwidth ratios were calculated, but we focused on those parameters showing robustness for detection and differentiation of the evaluated stress factors. As the results of the fluorescence measurements show, irrespective of nitrogen level, only a small number of the evaluated fluorescence ratios were suited to discriminate between healthy and inoculated leaves (Table 2). During the 3 day period of measurements, the half-bandwidth ratios B/G, G/R and G/FR revealed constant differences between inoculated and non-inoculated leaves. As shown for the B/G_*hbw* at 1 dai, powdery mildew lowered the ratio to

Table 2
Impact of nitrogen supply (N+, full-supply; N−, 40% of full-supply) and powdery mildew (PM) inoculation on selected fluorescence half-bandwidth ratios, determined from one to three days after inoculation (dai).

Fluorescence ratio	Experimental group	1 dai	2 dai	3 dai
B/G_*hbw*	N+	1.07 b	1.06 b	1.05 b
	N+/PM	1.00 a	0.95 a	0.94 a
	N−	1.07 b	1.05 b	1.04 b
	N−/PM	0.99 a	0.96 a	0.94 a
G/R_*hbw*	N+	1.95 a	1.97 a	1.97 a
	N+/PM	2.08 b	2.16 b	2.19 b
	N−	1.94 a	1.97 a	1.97 a
	N−/PM	2.09 b	2.14 b	2.16 b
G/FR_*hbw*	N+	1.51 a	1.52 a	1.52 a
	N+/PM	1.60 b	1.68 b	1.70 b
	N−	1.51 a	1.52 a	1.53 a
	N−/PM	1.61 b	1.67 b	1.69 b

Means of the fluorescence ratios for each evaluation day followed by the same letter do not differ significantly according to Duncan's multiple range test ($p \leq 0.05$; $n = 12$ for N+ and N−; $n = 16$ for N+/LR and N−/LR). *hbw* = half-bandwidth.

1.00 (N+/PM) and 0.99 (N−/PM) as compared to 1.07 in the non-inoculated leaves (Table 2). In contrast, ratios of G/R_*hbw* and G/FR_*hbw* were higher in *B. graminis* inoculated leaves compared to non-inoculated leaves. The G/R_*hbw* ratio for N+ and N− leaves at 1 dai were of 1.95 and 1.94, respectively, contrasting to 2.08 and 2.09 in the inoculated leaves (Table 2). During the following two days, the difference between healthy and inoculated leaves became more pronounced, and one day ahead of first visible infection symptoms (3 dai), the G/R_*hbw* ratio increased to 1.97 for both N+ and N−, 2.19 for N+/PM and 2.16 for N−/PM (Table 2).

Fig. 4C and D displays the time-course development of the B/G and R/FR amplitude ratios of the four experimental groups (N+, N−, N+/PM, N−/PM). During the whole evaluated period, plants supplied with adequate nitrogen showed the highest values for the B/G ratio, followed by the N− group, N+/PM, and finally N−/PM. On the first measuring day (1 dai) amplitude ratios of B/G for N+ and N− were of 3.73 and 3.55 respectively, whereas N+/PM and N−/PM leaves indicated significantly lower values of 3.19 and 3.06, respectively (Fig. 4C). During the following two days values decreased in all experimental groups but the difference between inoculated and non-inoculated leaves remained almost constant. On the last evaluation day (3 dai) values on N+ and N− were 3.48 and 3.31 and for inoculated ones, N+/PM and N−/PM, 2.76 and 2.66, respectively (Fig. 4D). Similarly, values of the ratio R/FR on healthy leaves remained at a comparable level, whereas infected leaves showed a significant increase. Already at 1 dai, significant differences between treatment groups were noted, and values were of 0.98 and 1.02 for N+ and N−, and 1.07 and 1.15 for N+/PM and N−/PM (Fig. 4D). At 3 dai values for N+ and N− were still at 0.96 and 1.00, whereas the groups N+/PM and N−/PM indicated ratios of 1.09 and 1.19, respectively (Fig. 4D).

Discussion

The objective of the current study was to evaluate the feasibility of spectral resolved fluorescence for simultaneous detection of slight N-deficiency and pathogen infection on the same leaves at a pre-symptomatic stage. Based on the fluorescence peaks in the blue, green, red and far-red regions as displayed in Fig. 1, amplitude, half-bandwidth, and amplitude-to-half-bandwidth ratios were established. The outcome was that several fluorescence ratios might be considered for detection and differentiation between the stressors. However, in contrast to the differentiation between N-deficiency and leaf rust (Table 1 and Fig. 4A, B), only a few ratios are suitable to differentiate N-deficiency and powdery mildew (Table 2 and Fig. 4C, D). In addition to the early detection of leaf rust and powdery mildew infection and slight nitrogen deficiency with the chlorophyll fluorescence, the amplitude ratio R/FR is also suited for simultaneous detection of both factors on the same leaves. Moreover, the blue–green fluorescence amplitude ratio (B/G) yields more precise results when distinguishing among the four experimental groups N+, N−, N+/pathogen, and N−/pathogen. As observed in both pathosystems, leaf rust and powdery mildew, the difference between values of the healthy and inoculated plants became more pronounced in the time-course of the infection's development.

It is well known that wheat plants grown under reduced N-supply exhibit lower chlorophyll levels compared to plants grown under full N supply (Cartelat et al., 2005), whereas the chlorophyll content can be estimated by handheld chlorophyll meters (Uddling et al., 2008). Our results revealed a reduction of chlorophyll content (Fig. 2) and an associated increase of the R/FR fluorescence ratio (Fig. 4), which is an inverse indicator of the chlorophyll content (Buschmann, 2007). A strong negative correlation coefficient ($r^2 = -0.86$) between the chlorophyll content and the amplitude ratio R/FR was established (*data not shown*). Moreover, our analyses showed a strong positive correlation coefficient ($r^2 = 0.90$) between the chlorophyll content and the B/G amplitude ratio (*data not shown*). Lichtenthaler et al. (1997) associated a decrease in the F440/F520 ratio with the change of chlorophyll content per leaf area, whereas the increase of blue fluorescence with decreasing chlorophyll and carotenoid content was discussed in terms of reduced re-absorption effects.

Experiments with barley and wheat grown under N-deficiency revealed close relations between the accumulation of phenolic metabolites and changes of chlorophyll content, and modifications in UV-induced fluorescence signature (Mercure et al., 2004; Cartelat et al., 2005). As a consequence of lower N supply in barley, the amount of total soluble phenolic compounds and the blue–green fluorescence increased (Mercure et al., 2004). In our study, the B/G fluorescence amplitude ratio decreased with less N supply (Fig. 4). This is consistent with the findings of (Belanger et al., 2006) who pointed out that the ratio F440/F520 revealed differences between potato plants fertilized with several nitrogen levels. In our study, the decrease of B/G values for plants grown under N-deficient conditions is explained by a combination of a small increase in blue fluorescence (2–3%) and a more pronounced increase in green fluorescence (6–7%).

General associations of the relationship between nitrogen status and fungal infection suggest that higher N supply increases the susceptibility of cereals to pathogens such as mildew and rusts (Walters and Bingham, 2007). Alternatively, low nitrogen levels were associated with higher amounts of phenols and reduced disease intensity (Cartelat et al., 2005), as phenols are known to play an important role in disease resistance (e.g. Herms and Mattson, 1992; Nicholson and Hammerschmidt, 1992; Vermerris and Nicholson, 2006). In addition, the synthesis and accumulation of such compounds depends on the time scale. As shown previously, slightly increased levels of bound and unbound hydroxycinnamic acid due to powdery mildew infection under low and medium N-supply were measured already 20 h after inoculation (Sander and Heitefuss, 1998). In the present studies with leaf rust and powdery mildew, the differences in B/G ratio between healthy and infected leaves became larger when infection was further developed (Fig. 4). Nevertheless, the difference between N−/pathogen and N+/pathogen leaves remained at the same level. As observed in our studies, absolute intensities of rust infected leaves increased on average from 4% (dai 2) to 23% (dai 4) for the blue and from 11% (dai 2) to 55% (dai 4) for the green fluorescence as compared to the non-inoculated tissue, irrespective of nitrogen supply.

Alternatively, re-absorption effects of blue fluorescence light by chlorophyll (Lang et al., 1991), as well as a possible shielding effect of the excitation light by phenolics located in the epidermis (Chaerle and Van Der Straeten, 2000) should be considered. The observed increase of the R/FR ratio in our experiments (Fig. 4) indicates a decrease in chlorophyll content in plants inoculated with leaf rust or powdery mildew (Buschmann, 2007), which is in accordance with Lorentzen and Jensen (1989) and Owera et al. (1981).

As reported by several authors (McMurtrey et al., 1994; Heisel et al., 1996; Mercure et al., 2004), several computed fluorescence ratios the F440/F685, F440/F740, F525/F685 and F740/F685 revealed differences between maize plants fertilized with 100% or with 75% or less nitrogen. The ratios F440/F690 and F440/F740 proved to be more sensitive to nutrient deficiencies than the F690/F740 ratio (Heisel et al., 1996; Lichtenthaler et al., 1997). Our results did not confirm this for plants grown under N-deficiency for a comparatively very short period. These fluorescence parameters seem to be more suited to reveal early pathogen infection irrespective of the nitrogen status of the plants, as observed for

amplitude and half-bandwidth ratios in the leaf rust experiment (Table 1), and of half-bandwidth ratios in the powdery mildew trials (Table 2). In the experiment with leaf rust, especially the amplitude ratios G/R and G/FR as well as the FR* amplitude to half-bandwidth ratio showed a strong effect of pathogen infection during two to four days after inoculation (Table 2). Nevertheless, changes in these ratios in response to nitrogen deficiency are expected under progressed and more pronounced limitations as compared to the slight deficiency conditions in our experiment.

Comparing both leaf rust and powdery mildew pathosystems, the difference between infected and healthy leaves for the parameters B/G and R/FR amplitude ratio were smaller for leaf rust than for powdery mildew (Fig. 4). In addition, the time-course development of the fluorescence signals was more pronounced for leaf rust. Nevertheless, both fluorescence parameters allowed differentiation among the experimental groups N+, N−, N+/LR or PM, and N−/LR or PM. Going forward, improved statistics and raw data analysis under consideration of classification algorithms such as Decision Trees, Naive Bayes, Artificial Neural Networks, Logistic Regression and Support Vector Machines (SVMs) might render a more precise classification. Preliminary results indicate that SVMs yield a precise discrimination of healthy and infected leaves (Römer et al., 2010). Ongoing studies will clarify whether such data evaluation tools can support and improve robust differentiation between simultaneous occurring of biotic and abiotic stresses.

Conclusion

The fluorescence signature measured between 370 and 800 nm is a useful tool when addressing the challenge of discrimination between biotic and abiotic stress factors. The focus on blue–green fluorescence yields important additional information for a more precise discrimination as compared to previous approaches with chlorophyll fluorescence. The amplitude ratio B/G as well as R/FR revealed to be well suited to distinguish among N-full-supply, N-deficiency, N-full-supply + pathogen, and N-deficiency + pathogen. In addition, several fluorescence ratios facilitated early detection of leaf rust or powdery mildew infection irrespective of the plants' nitrogen status.

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