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# Multicolour fluorescence imaging of sugar beet leaves with different nitrogen status by flash lamp UV-excitation

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## Abstract

Fluorescence images of leaves of sugar beet plants (*Beta vulgaris* L. cv. Patricia) grown on an experimental field with different fertilisation doses of nitrogen [0, 3, 6, 9, 12, 15 g(N) $m^{-2}$] were taken, applying a new multicolour flash-lamp fluorescence imaging system (FL-FIS). Fluorescence was excited by the UV-range (280–400 nm, $\lambda_{max}$ = 340 nm) of a pulsed Xenon lamp. The images were acquired successively in the four fluorescence bands of leaves near 440, 520, 690, and 740 nm ($F_{440}$, $F_{520}$, $F_{690}$, $F_{740}$) by means of a CCD-camera. Parallel measurements were performed to characterise the physiological state of the leaves (nitrogen content, invert-sugars, chlorophylls and carotenoids as well as chlorophyll fluorescence induction kinetics and beet yield). The fluorescence images indicated a differential local patchiness across the leaf blade for the four fluorescence bands. The blue ($F_{440}$) and green fluorescences ($F_{520}$) were high in the leaf veins, whereas the red ($F_{690}$) and far-red ($F_{740}$) chlorophyll (Chl) fluorescences were more pronounced in the intercostal leaf areas. Sugar beet plants with high N supply could be distinguished from beet plants with low N supply by lower values of $F_{440}/F_{690}$ and $F_{440}/F_{740}$. Both the blue-green fluorescence and the Chl fluorescence rose at a higher N application. This increase was more pronounced for the Chl fluorescence than for the blue-green one. The results demonstrate that fluorescence ratio imaging of leaves can be applied for a non-destructive monitoring of differences in nitrogen supply. The FL-FIS is a valuable diagnostic tool for screening site-specific differences in N-availability which is required for precision farming.

*Additional key words*: *Beta vulgaris* L.; blue-green fluorescence; chlorophyll fluorescence; fluorescence ratios; nitrogen nutrition; photosynthetic activity; protein; sugars; yield.

## Introduction

Farmers need profound knowledge on the local differences in soil and plant conditions occurring in present-day large fields to optimise the crop yield. Different usage of fertiliser, herbicides, and other plant treatments are of ecological and economic importance. Accurate, reliable, and objective methods using high-resolution spatial information are required for modern "precision farming" (Sylvester-Bradley *et al.* 1999).

The determination of appropriate nitrogen (N) supply to plants is of special interest because N is the crucial mineral nutrient for plant growth and high crop yield. One part of the essential N is fixed in the organic matter of the soil (mineralised N, $N_{min}$), and can be mobilised during the vegetation period depending on weather conditions. However, the major part of N has to be supplied by fertilisers before sowing and during the

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*Abbreviations*: CCD - charged coupled device; Chl - chlorophyll; DM – dry matter; $F_{440}$, $F_{520}$, $F_{690}$, $F_{740}$ - fluorescence intensity at the wavelength numbers [nm]; $F_0$ and $F_p$ - minimum (open reaction centres) and maximum (closed reaction centres) of Chl *a* fluorescence of dark adapted leaves; $F_s$ - steady state Chl *a* fluorescence in irradiated leaves (*ca.* 5 min); $F_d$ - decrease of Chl *a* fluorescence from the maximum $F_p$ to the steady state $F_s$; $F_v$ - variable Chl fluorescence ($F_p - F_0$); $F_v/F_p$ - optimal quantum yield of PS2; $F_v/F_0$ - ratio of quantum yield of PS2; N - nitrogen; FIS - fluorescence imaging system; FL - flash lamp; FM – fresh matter; PS – photosystem; $R_{Fd}$ - ratio of variable Chl fluorescence decrease $(F_p - F_s)/F_s$; $T_{1/2}$ - time of half rise from $F_0$ to $F_p$; Xe – xenon lamp; *SPAD*-value – value obtained by the portable chlorophyll meter *SPAD-502* developed by the *Soil-Plant Analysis Development (SPAD) Section of Minolta Camera.*

growth of young plants. A site-specific N-application is needed not only for a high yield profit, but also for drinking water protection, since a surplus of N can easily be washed out as nitrate to the ground water when it is not incorporated in organic matter.

Conventionally, the plant's N-supply for the appropriate N-applications is obtained by chemical plant analysis of the N-content, which is a time-consuming process and destroys the plant material examined. Producers and scientists are therefore seeking accurate methods to estimate N-supply that are non-destructive and have the potential to be automated and applied in the field.

Most of the N in the leaf is allocated to photosynthetic function. 50-80 % of the total leaf N is required for the performance of photosynthesis and a high proportion of it is incorporated into Chl and the pigment proteins of thylakoids. Therefore, the greenness of leaves is positively correlated with their N-supply (Evans 1989). N-management in sugar beet production is a difficult task, because there must be found a balance between sugar beet quantity and quality. Conditions for a large leaf area of sugar beet plants are favoured by high N-applications, but then the quality of the beet is impaired, because the beets contain many soluble proteins and nitrate which bind sugar in the sugar processing and thus reduce the final sugar yield (Bornscheuer 1986).

While reflectance measurements of plants in the near infra-red spectral region are already being applied to detect N-supply differences (*e.g.*, Filella *et al.* 1995, Ma *et al.* 1996, Moraghan 1998, Vouillot *et al.* 1998) and recently also tested in a commercial tractor-mounted sensing system (Reusch 1997 and Hydro N-senor, *Hydro Agri*, Hanninghof, Germany) during fertiliser application, methods using the fluorescence signatures of crop plants are still developed. Fluorescence provides much deeper information on the plant condition than reflectance because it is more plant specific and directly correlated to the photosynthetic activity *via* Chl *a* fluorescence, one of the de-excitation processes besides heat and photochemistry. Changes in leaf biochemistry can be recognised by measuring Chl fluorescence already at an early stage of stress of plants, when stress effects or damage are not yet visually detectable (*e.g.*, Lichtenthaler and Rinderle 1988). The large-scale practical application of Chl fluorescence measurements in the field is hampered because it is difficult to access the weak signal under ambient irradiance. In addition, for excitation of Chl fluorescence a strong light source, like expensive UV-lasers, is needed (Lichtenthaler *et al.* 1996).

Fluorescence emissions of green leaves, when excited by UV-radiation, consist of two different fluorescence types: (*1*) the blue-green fluorescence with a maximum in the blue ($F_{440}$) and a shoulder in the green ($F_{520}$), originating primarily from fluorophores such as ferulic acid and flavonoids in the cell walls of the epidermis and leaf veins (Morales *et al.* 1996, Lichtenthaler and Schweiger 1998, Johnson *et al.* 2000), and (*2*) the Chl *a* fluorescence from chloroplasts of the green mesophyll cells, with maxima in the red (near 690 nm) and far-red (near 740 nm) (Lichtenthaler and Rinderle 1988, Krause and Weis 1991, Maxwell and Johnson 2000). The characteristics of UV-excited fluorescence signatures of leaves were reviewed by Buschmann and Lichtenthaler (1998) and Cerovic *et al.* (1999).

Point measurements of Chl *a* fluorescence, especially of the Chl fluorescence induction kinetics, are a well-established method to understand the basics of photosynthesis (*e.g.*, Lichtenthaler *et al.* 1981, Lichtenthaler 1987, Strasser *et al.* 1995, Rosema *et al.* 1998, Richter *et al.* 1999, Samson *et al.* 1999). They also detect the changes and adaptation of photosynthetic function in plants under different growth conditions, environmental and nutrient stress, as well as attack by pathogens and pests (*e.g.*, Lichtenthaler and Rinderle 1988, Araus *et al.* 1998, Strasser and Tsimilli-Michael 1998, Fracheboud *et al.* 1999, Georgieva and Lichtenthaler 1999, Jalinek *et al.* 1999, Tyystjärvi *et al.* 1999, Barósi *et al.* 2000, Shangguan *et al.* 2000). However, Chl fluorescence signatures often vary within a single leaf (*e.g.*, Buschmann 1981, Šesták and Šiffel 1997) and such local differences (Lang *et al.* 1996) are difficult to detect even with multiple point data measurements.

With the progress in technology and the access to sensitive high-resolution CCD-cameras, which by means of gated intensifiers can catch even intensities in the photon range, low signal intensities such as the fluorescence emissions of the leaves can be visualised by fluorescence images and analysed pixel by pixel over the whole leaf area (resolution of several ten thousands of pixels per leaf). Diverse fluorescence imaging systems were developed in laboratories that use spatial information of the plant components by recording the maxima of the four fluorescence emission bands (multicolour imaging) and their changes influenced by stress of different origin (Lang *et al.* 1994, Lichtenthaler and Miehé 1997, Buschmann and Lichtenthaler 1998, Buschmann *et al.* 2000).

Encouraging attempts have been made with laboratory systems (Heisel *et al.* 1996, 1997, Sowinska *et al.* 1998) to discriminate among field plant leaves with different N-content with UV-laser-induced fluorescence imaging. These attempts have led to the development of a mobile laser-FIS system for N-monitoring of plants in the field that is being tested in a pioneer project (Sowinska *et al.* 1999). Since laser fluorescence imaging systems are very costly, we developed a low-cost laboratory UV-flash-lamp fluorescence imaging system (FL-FIS). With the application of this new FL-FIS device to study field-grown sugar beet we wanted to prove if fluorescence imaging can satisfactorily distinguish between a different N-supply of sugar beet plants.

## Materials and methods

**Plants**: Sugar beet plants (*Beta vulgaris* L. cv. Patricia) were grown on an experimental field of the State Agricultural Testing and Research Station (LUFA) Augustenberg at Ladenburg, Germany. Six different N levels were applied using a fertiliser ($NH_4NO_3$ + $CaCO_3$ with 27 % N): 0, 3, 6, 9, 12, and 15 g(N) $m^{-2}$ termed N0, N3, N6, N9, N12, and N15, respectively. This corresponds to 0, 30, 60, 90, 120, and 150 kg(N) per hectare, respectively. Concerning the total N-supply of the plants the residual organic N in the soil ($N_{min}$) of 1.5–2.0 g(N) $m^{-2}$ in each test plot has to be added. Leaf samples were taken on different occasions, and for fluorescence imaging on October 10, 1999 when the sugar beets were harvested. Young expanded leaves of the inner part of the leaf rosette were used. Leaves taken in the field were placed in plastic bags inside a portable ice chest, transported to the laboratory, and measured within a few hours.

**Chls and carotenoids** of the leaves were determined spectrophotometrically from leaf extracts in 100 % acetone using the re-determined extinction coefficients and equations of Lichtenthaler (1987). The same leaf disks (9 mm diameter) that had been used before for measuring the Chl fluorescence induction kinetics were extracted. The Chl content was also characterised in a non-destructive way using a chlorophyll meter (*SPAD-502, Minolta*, Osaka, Japan). The *SPAD*-values indicate the relative amount of Chl present in plants. They are based on the amount of radiation transmitted by the leaf in two wavelength regions in which the absorbance of Chl is high (650 nm) and low (940 nm) (cf. Markwell *et al.* 1995, Bullock and Anderson 1998, Schepers *et al.* 1998).

**Other analyses**: Sugar beet yield and the chemical composition of the leaves and beets were determined by standard methods. N and raw protein contents were measured with a combustion analyser system (*FP 2000, LECO*, St. Joseph, U.S.A.), nitrate by ion chromatography as described by Baffler (1997b), and invert sugars as described by Baffler (1997a).

**Chl fluorescence induction kinetics** (Kautsky effect) were measured simultaneously at 690 and 735 nm using the compact, portable, two-wavelength Chl fluorometer (*BUKA, CFM-636973, Opti-Sens*, Budapest, Hungary) (Subhash *et al.* 1999, Barósi *et al.* 2000) constructed on the basis of the Karlsruhe laboratory LITWaF (laser-induced two-wavelengths fluorometer) (Lichtenthaler and Rinderle 1988). The leaves were pre-darkened for 20 min. Chl fluorescence was excited with a 10 mW laser diode ($\lambda_{max}$= 635 nm; actinic irradiance 500 µmol $m^{-2}$ $s^{-1}$) in the adaxial leaf side of leaf disks (diameter 9 mm). Values were stored, characteristic parameters of the Chl fluorescence induction automatically calculated and then transferred to a personal computer for further processing.

**Fluorescence imaging**: Images of sugar beet leaves were measured with the new multicolour flash-lamp fluorescence imaging system (FL-FIS) developed by Botany II, Karlsruhe (Lichtenthaler *et al.* 2000). The latter is based on the laser-FIS (Lang *et al.* 1994, Lichtenthaler *et al.* 1996, Heisel *et al.* 1999). For the FL-FIS a less expensive UV excitation source with a flashed Xenon (Xe) arc lamp (*FX300UV, ILC Technology*, Sunnyvale, USA) was chosen. The excitation was limited to the UV-A range by a broad-band filter (*DUG 11, Schott*, Mainz, Germany, 280-400 nm, $\lambda_{max}$ = 340 nm). Images were taken by a digitised CCD camera (*Photonetics*, Kehl, Germany) with a gated intensifier (variable gain up to 3 000 W $W^{-1}$). Imaging in the four fluorescence bands (440, 520, 690, and 740 nm) was carried out successively using interference filters in the respective bands (*Oriel*, France, bandwidth 10 nm) installed in a filter wheel behind the camera lens. The flashes of the Xe lamp had a duration of 20 µs with a jitter of ±15 µs. In order to catch the entire signal a gating time of 340 µs with a frequency of 16 Hz was applied. Image acquisition, camera control, synchronisation of flashes of the Xe lamp with the camera, and image processing was carried out with a personal computer *via* an *RS 232* interface card and special custom made software (*ARP-Camille*, version *1.05, Photonetics*, Kehl, Germany). A frame grabber card (*Oculus F64, Coreo*, Québec, Canada) was used to grab the image from the camera. Images were acquired at a distance of 0.5 m and consisted of 565 lines with 754 pixels per line. The camera acquired and stored each readout on 8 bits, but the images were accumulated up to 16 bits in the PC by means of the interface card and the software. All images were automatically corrected for the non-uniformity of the excitation radiation during acquisition by a reference fluorescence image and the attenuation factors of the interference filters. As reference the $F_{440}$ image of a white paper sheet was used.

To exclude changes of the Chl fluorescence due to the photosynthetic induction kinetics (Kautsky effect) during the measurements, the sugar beet leaves where pre-irradiated with "white light" for 5 min (800 µmol $m^{-2}$ $s^{-1}$). Fluorescence of the leaves was excited and measured at the upper leaf side at a distance of *ca.* 0.5 m. The final fluorescence image at each of the four fluorescence bands consisted of 200 fluorescence readouts accumulated under ambient irradiance and corrected by subtraction of 200 readouts of the ambient irradiance with the flash lamp turned off. A binary mask was used to exclude background pixels and to limit the fluorescence image to the actual leaf surface. Fluorescence intensities of the images were displayed in 8 false colour steps. Arithmetic calculations were performed to obtain pixel by pixel ratio

images of the whole leaf area. A profile could be chosen to further characterise the fluorescence properties of the leaves across the transversal leaf axis. Mean values of all leaf pixels (>250 000 per leaf) were calculated in the four fluorescence bands and for the four fluorescence ratios for seven leaves of each N-supply condition. The frequency distribution of the pixel values can be shown as a leaf histogram. The variation of the pixel means between the seven leaves in the blue and green fluorescence was lower (±10 % or less) than that for the red and far-red Chl fluorescence (±20 % or less). The variation of the pixel means between the leaves of one N-supply condition amounted for the fluorescence ratios blue/green to <10 %, blue/red and blue/far-red to <20 %, and red/far-red to <5 %.

## Results and discussion

**Nitrogen, sugar, and yield**: The sugar beet plants were subjected to conventional plant analysis in the laboratory. The N-content of the leaves increased gradually from low to high N-supply (Table 1). Leaves of the high N-plot N15 contained 85 % more N than those of the low N-plot N0. An adequate N-content of mature sugar beet leaves is between 4.0-6.0 % N of leaf dry mass (Bergmann 1992). The recommended N-supply for sugar beet plants in the experimental field was 10-12 g(N) $m^{-2}$. Thus, leaves from the plant plots N0-N9 showed a decreasing N-deficiency. With the rise in total N from N0 to N15, the amounts of nitrate stored in the leaves were increased by more than forty times. Up to plot N6, almost no N was stored as nitrate. An elevated nitrate content at harvest, as in the plot N15, indicates an imbalance between N-supply and N-demand for growth, and is uneconomical in terms of N-utilisation (Marschner 1995).

When looking at the harvested crop—the sugar beets—their content of invert sugars was independent of the N-supply, but their protein content, which was constant up to N6, increased from N9 to N15. Usually, the beet yield should increase parallel to the N-fertilisation (Bornscheuer 1986). The highest beet yield was found for the N9 plot with 5.73 kg $m^{-2}$ (573 dt/ha), but was lower at a lower and higher N-supply. The reduced beet yield for N12 and N15 may be due to the

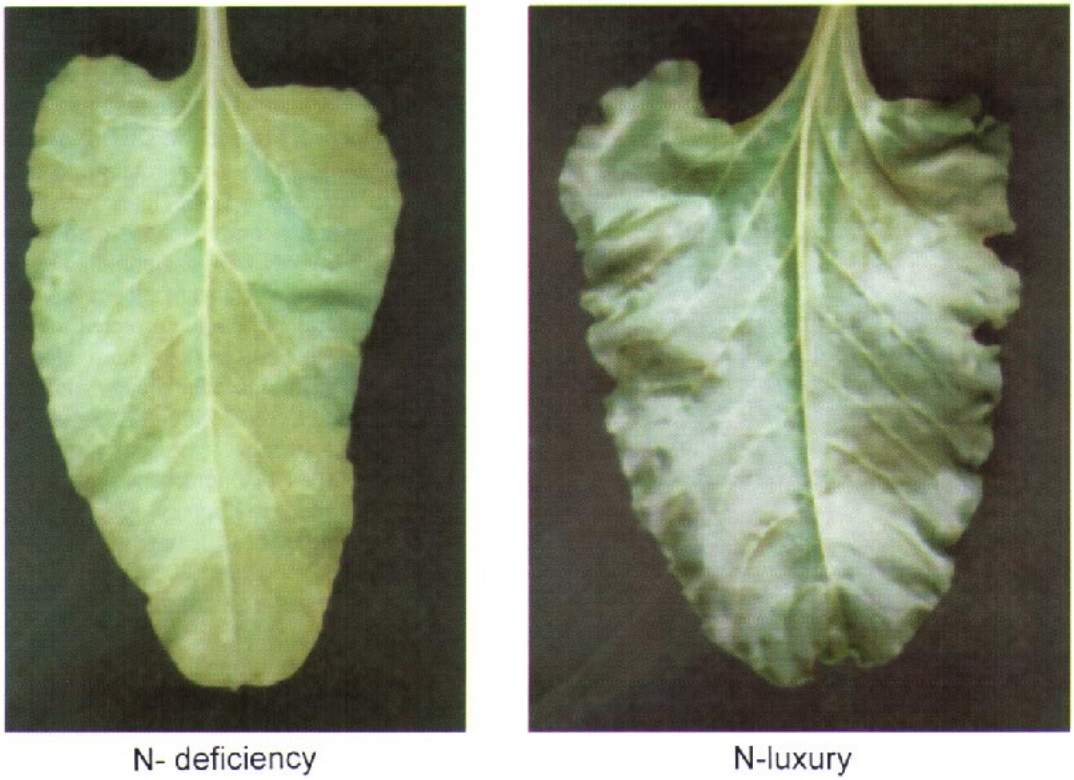


Fig. 1. Photographs of a sugar beet leaf grown with no nitrogen supplied [N0 = 0 g(N) $m^{-2}$; N-deficiency] and a leaf grown with a high amount of nitrogen supplied (N15; N-luxury). The leaves were excised at beet harvest. The fluorescence images and ratio images of these leaves are presented in Fig. 4, and a horizontal leaf profile is given in Fig. 5.

Table 1. Content of total nitrogen (N) and nitrate ($NO_3^-$) of sugar beet leaves and yield of beet as well as the quality parameters of invert sugars and protein of beets. Sugar beet plants were grown with the following nitrogen supply added as fertiliser: N0, N3, N6, N9, N12, and N15 [figures give g(N) $m^{-2}$]. Leaf nitrogen and nitrate content were determined from the leaves of the plant plot from which the fluorescence images were taken, whereas beet yield and quality parameters are the mean of four repeated plant plots of each nitrogen supply. The plants are grouped for low (N0, N3), medium (N6, N9), and high (N12, N15) N supply.

| | Leaf N [%DM] | $NO_3^-$ [mg $kg^{-1}$(FM)] | Sugar beet Invert sugars [%DM] | Protein [%DM] | Yield [kg $m^{-2}$] |
|---|---|---|---|---|---|
| N0 | 2.72 | 7 | 76.0 | 2.9 | 3.96 |
| N3 | 2.87 | 7 | 77.5 | 2.8 | 4.10 |
| N6 | 3.25 | 10 | 77.2 | 2.9 | 4.84 |
| N9 | 3.86 | 68 | 77.0 | 3.3 | 5.73 |
| N12 | 4.27 | 107 | 77.1 | 3.9 | 5.37 |
| N15 | 5.05 | 299 | 76.0 | 4.3 | 5.00 |

Table 2. Differences in the contents of chlorophylls ($a+b$) and carotenoids ($x+c$) [mg $m^{-2}$(leaf area)] as well as the pigment ratios Chl $a/b$ and $(a+b)/(x+c)$ and *SPAD*-values of sugar beet leaves grown with the following nitrogen supply added as fertiliser: N0, N3, N6, N9, N12, and N15 [figures give g(N) $m^{-2}$]. Mean values from 7 determinations of 7 separate leaves of each N-supply at beet harvest. The *SPAD*-values are based on 10 determinations per leaf. Mean values of the pigment content and the *SPAD*-values with different letters show a significant difference ($p \leq 0.01$) between the various nitrogen-supplies. [1, 2, 3]Standard deviations maximal ± 3, 5, or 17 %, respectively.

| | $(a+b)$ | $(x+c)$ | $a/b$[1] | $(a+b)(x+c)$[2] | *SPAD*[3] |
|---|---|---|---|---|---|
| N0 | 271±43 [a] | 73.3±9.0 [a] | 3.14 [a] | 3.70 [a] | 32.9 [a] |
| N3 | 278±56 [a] | 67.6±14.0 [a] | 3.21 [a] | 4.13 [b] | 34.6 [a] |
| N6 | 407±78 [b] | 99.8±15.0 [b] | 3.30 [a] | 4.05 [b] | 44.1 [b] |
| N9 | 414±35 [b] | 91.3±9.0 [b] | 3.26 [a] | 4.56 [c] | 45.5 [b] |
| N12 | 518±98 [c] | 117.0±19.0 [c] | 3.03 [b] | 4.43 [c] | 54.5 [c] |
| N15 | 585±25 [c] | 123.0±8.0 [c] | 3.03 [b] | 4.77 [c] | 55.1 [c] |

extreme dry weather from July until the beginning of September 1999, the main growth phase of beets. Water shortage is particularly harmful for plants with a high N-supply, as being equipped with a huge total leaf area and a small root system, they need a lot of water for their growth. For sugar beet plants the water demand is high in general (Bornscheuer 1986). At times of water shortage the huge leaf area itself needs to be maintained, otherwise leaves are partially discarded and less assimilates are transported to the growing beets (Röver 1998).

**Photosynthetic pigments**: With increasing N-supply from N0 to N15, the content of leaf Chl ($a+b$) rose by more than 100 % and that of total carotenoids ($x+c$) increased by 65 % (Table 2). Also the SPAD-values, an indirect indicator of the Chl content, increased in the same range of N-supply by 67 %. This result is in agreement with values in the literature on N-deficiency (barley: Hák *et al.* 1993, sunflower: Ciompi *et al.* 1996, bean: Lima *et al.* 1999, wheat: Shangguan *et al.* 2000). The differences in pigment concentration between leaves of N0 and N15 plants were also visible by eye (Fig. 1). Both leaves looked healthy, but the lower Chl content of the N0-leaf resulted in a light-green appearance as compared to the dark-green leaf of the N15 plots. There is a general tendency for the values of Chl $a/b$ to be higher at low and medium N-supply as compared to the plants with high N-supply (maize and barley: Lichtenthaler and Rinderle 1988). Lower values of Chl $a/b$ indicate the presence of more light-harvesting Chl complexes of LHC2 (Lichtenthaler *et al.* 1982, Lichtenthaler 1987). The leaves from sugar beet plants with lower N-suply levels showed the same tendency for higher Chl $a/b$ values throughout the whole growing season.

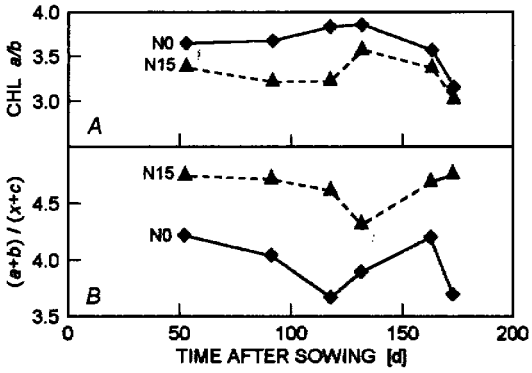


Fig. 2. Development of the pigment ratios Chl $a/b$ (*A*) and Chls to carotenoids $(a+b)/(x+c)$ (*B*) at low (N0) and high (N15) N-supply of sugar beet plants during the vegetation period. The pigments were determined on May 29, July 7, August 2 and 16, September 30, and October 19. Means of 10 determinations from the 10 youngest fully developed leaves. The differences between low and high N-supply plants are significant ($p \leq 0.01$).

The ratio of Chls to total carotenoids, $(a+b)/(x+c)$, rose with increasing N-availability of the sugar beet plants (Table 2). It was higher at higher N-supply (N15 *versus* N0) throughout the vegetation period (Fig. 2*B*). This ratio is an indicator of the greenness of plants (Lichtenthaler 1987); under stress conditions and senescence, Chls break down faster than carotenoids and $(a+b)/(x+c)$ decreases considerably. Moreover, sun leaves with sun-type chloroplasts and less LHC2 have *ca.* 20 to 30 % lower values for $(a+b)/(x+c)$ than shade leaves (*e.g.*, Lichtenthaler and Rinderle 1988). Thus the sugar beet leaves with low N-supply (N0 to N6) and rather sun-type like chloroplasts showed the expected lower $(a+b)/(x+c)$ between 3.7 and 4.1. This is in agreement with reports on N-deficient barley (Hák *et al.* 1993) and maize (Khamis *et al.* 1990).

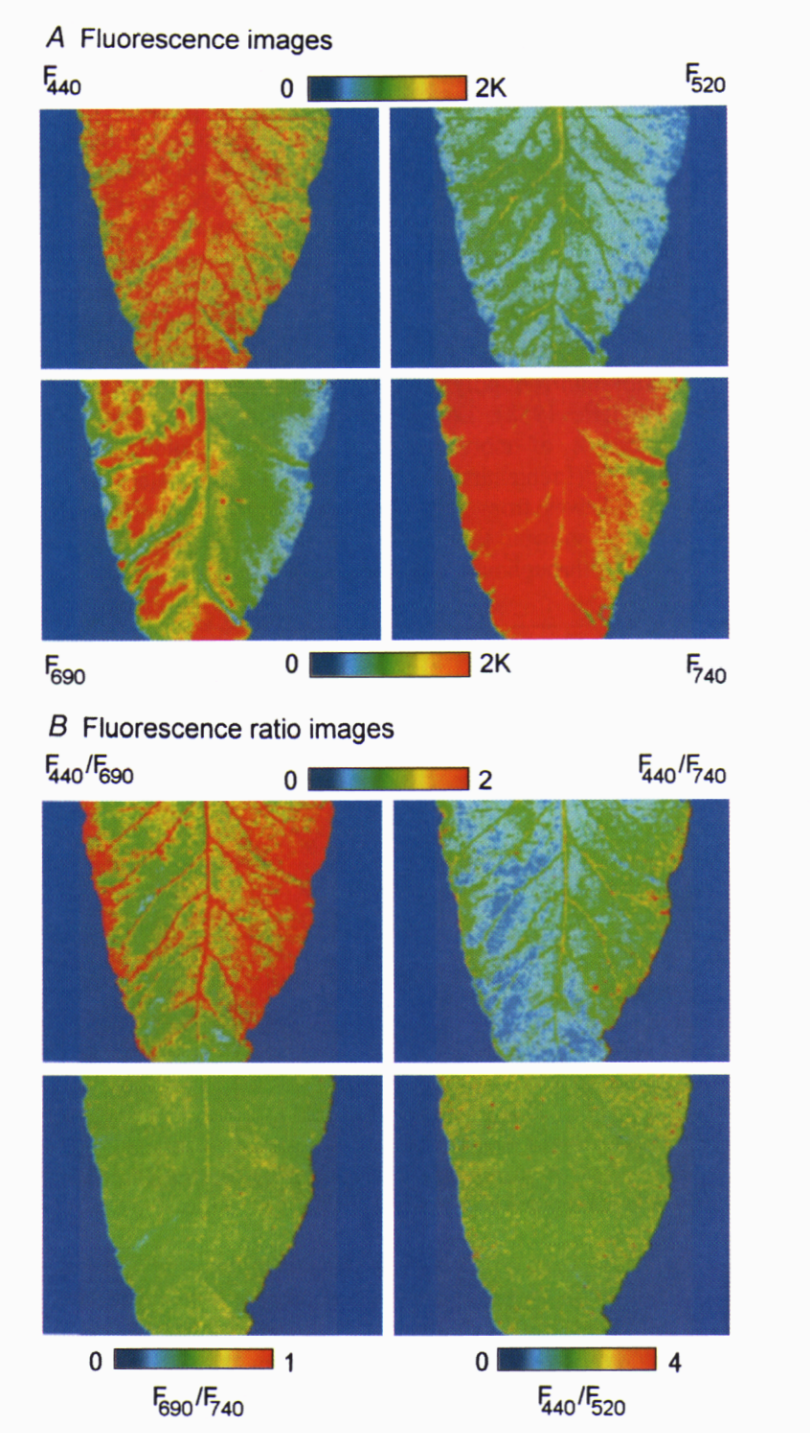


Fig. 3. Fluorescence images (*A*) of the four fluorescence bands blue ($F_{440}$), green ($F_{520}$), red ($F_{690}$), and far-red ($F_{740}$) as well as the fluorescence ratio images (*B*) blue/red ($F_{440}/F_{690}$), blue/far-red ($F_{440}/F_{740}$), red/far-red ($F_{690}/F_{740}$), and blue/green ($F_{440}/F_{520}$) of a leaf from a sugar beet plant grown with 12 g(N) $m^{-2}$ (recommended supply) measured at beet harvest. Images are presented in false colours with increasing fluorescence intensities from dark blue (low) to red (high), as indicated in the respective scales (K = 1 000 fluorescence intensity units). The images are based on more than 250 000 pixels per leaf.

**Photosynthetic activity was measured *via* Chl fluorescence induction kinetics** using the *BUKA* fluorimeter, where Chl fluorescence is excited by a red laser diode ($\lambda_{exc}$ = 635 nm). In contrast to UV-excitation, which can be filtered and absorbed in the epidermis cells of leaves by the soluble flavonols and cinnamic acids, red excitation radiation passes the epidermis cells, unhindered by flavonols, and can induce a strong Chl fluorescence

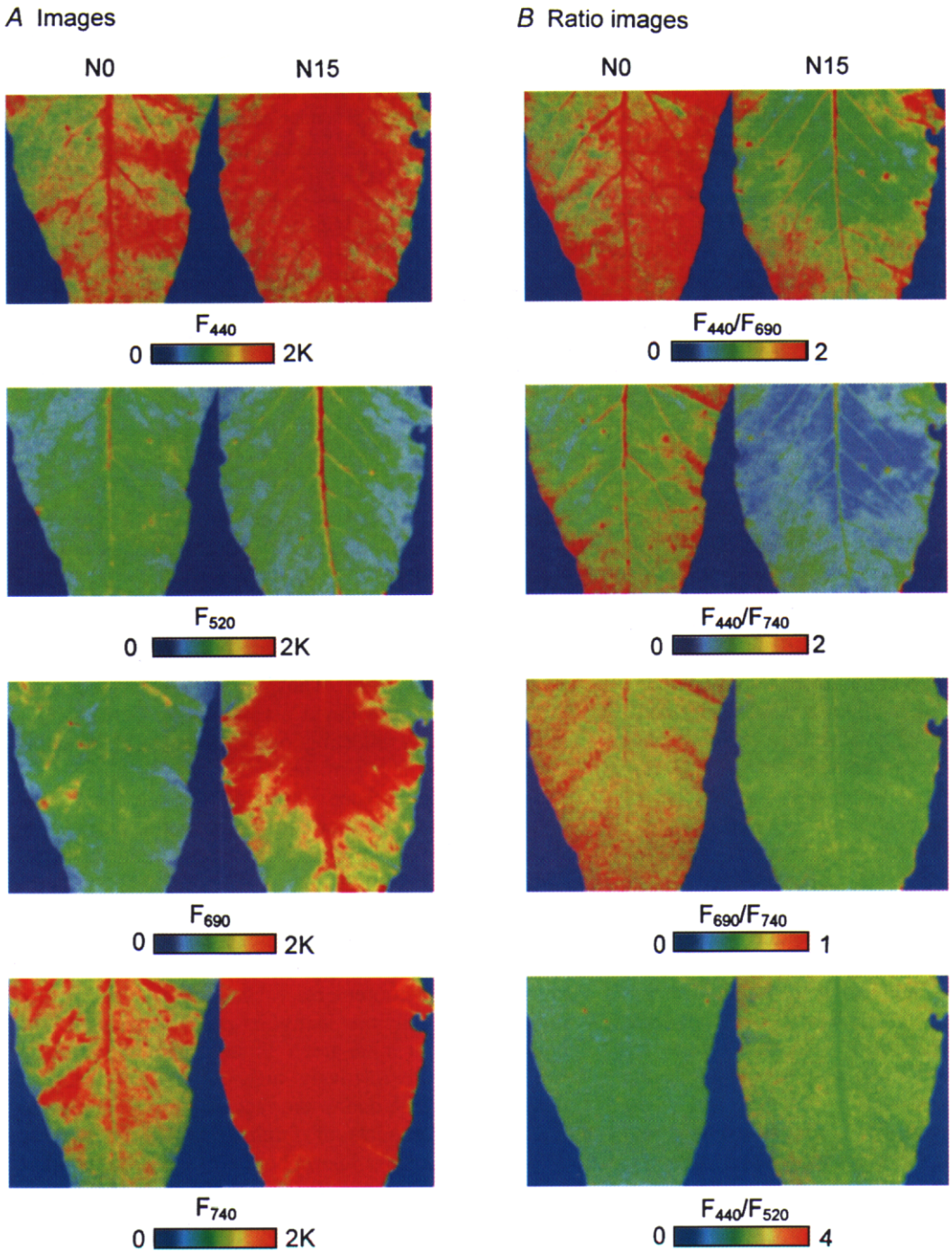


Fig. 4. Fluorescence images (*A*) and fluorescence ratio images (*B*) of a leaf of a sugar beet plant grown with no nitrogen supplied [N0 = 0 g(N) $m^{-2}$; N-deficiency] and of a plant grown with a high amount of nitrogen supplied (N15; N-luxury). The leaves were excised at beet harvest. The fluorescence images are shown for the four fluorescence bands: blue ($F_{440}$), green ($F_{520}$), red ($F_{690}$), and far-red ($F_{740}$). The fluorescence ratio images are given for the ratios blue/green ($F_{440}/F_{520}$), blue/red ($F_{440}/F_{690}$), blue/far-red ($F_{440}/F_{740}$), and red/far-red ($F_{690}/F_{740}$). The images are presented in false colours with increasing fluorescence intensities from dark blue (low) to red (high), as indicated in the respective scales (K = 1 000 fluorescence intensity units). The images are based on more than 250 000 pixels per leaf.

signal in the Chl containing mesophyll cells. The basic Chl fluorescence parameters $F_0$, $F_p$, $F_s$, $F_v$, and $F_d$ decreased by up to 20 % with increasing N-supply from N0 to N15 (Table 3). The decrease in these Chl fluorescence emission parameters can mainly be explained by higher re-absorption of the emitted

Table 3. Parameters of the chlorophyll *a* fluorescence induction kinetics (Kautsky effect) measured with the *BUKA* fluorimeter (red laser diode excitation) of pre-darkened sugar beet leaves grown with the following nitrogen supply added as fertiliser: N0, N3, N6, N9, N12, and N15 [figures give g(N) $m^{-2}$]. The Chl fluorescence kinetics were measured from the onset of irradiation ($F_0$) *via* the fluorescence peak ($F_p$) to the steady state fluorescence ($F_s$) reached after 5 min. $F_v$ is the variable fluorescence ($F_p - F_0$) and $F_d$ the fluorescence decrease from $F_p$ to $F_s$ ($F_p - F_s$). Means from seven different leaves of each nitrogen supply at beet harvest.

| | N0 | N3 | N6 | N9 | N12 | N15 |
|---|---|---|---|---|---|---|
| $F_o$ | 1.41±0.10 | 1.52±0.30 | 1.21±0.10 | 1.30±0.10 | 1.11±0.10 | 1.13±0.10 |
| $F_p$ | 6.28±0.50 | 6.34±0.60 | 5.25±0.30 | 5.53±0.50 | 4.88±0.50 | 4.95±0.30 |
| $F_s$ | 1.62±0.20 | 1.69±0.50 | 1.39±0.10 | 1.51±0.30 | 1.25±0.10 | 1.26±0.10 |
| $F_v$ | 4.87±0.40 | 4.72±0.40 | 4.04±0.30 | 4.23±0.40 | 3.77±0.40 | 3.81±0.30 |
| $F_d$ | 4.71±0.40 | 4.58±0.30 | 3.89±0.20 | 4.06±0.40 | 3.65±0.30 | 3.69±0.40 |
| $T_{1/2}$ [s] | 21.70±5.60 | 18.30±2.00 | 13.00±2.10 | 15.40±2.10 | 15.30±3.50 | 16.40±1.20 |
| $F_v/F_p$ | 0.78±0.01 | 0.75±0.03 | 0.77±0.01 | 0.77±0.01 | 0.77±0.01 | 0.77±0.01 |
| $F_v/F_0$ | 3.46±0.20 | 2.97±0.40 | 3.35±0.20 | 3.27±0.2± | 3.39±0.20 | 3.36±0.20 |
| $R_{Fd}$ | 2.91±0.20 | 2.71±0.30 | 2.80±0.20 | 2.69±0.3± | 2.92±0.20 | 2.93±0.10 |
| $F_{690}/F_{740}$ | 0.75±0.04 | 0.75±0.05 | 0.69±0.04 | 0.69±0.03 | 0.66±0.02 | 0.62±0.02 |

Chl fluorescence connected with increase in Chl content of the leaves (Lichtenthaler and Rinderle 1988, Babani and Lichtenthaler 1996). This is due to the fact that the red Chl fluorescence emission band ($F_{690}$) overlaps with the absorption bands of the in vivo leaf Chl (Gitelson et al. 1999). The half rise time from $F_0$ to $F_p$, $T_{½}$, showed the highest values for the leaves in the low N-supply plot, but no differences between the medium and high N-plot. $T_{½}$ indicates the pool size of the available electron acceptor (oxidised quencher Q) on the reducing side of PS2 (van Gorkom 1986). $T_{½}$ is low, when the total amount of quencher Q is small, the proportion of oxidised Q per total Q is low or when the efficiency of radiation absorption is high as in shade-type chloroplasts. The variable Chl fluorescence ratios $F_v/F_p$, $F_v/F_0$, and $R_{Fd}$, which indicate photosynthetic quantum conversion (Babani and Lichtenthaler 1996), showed no differences between the N-supplies indicating a good quantum conversion (Lichtenthaler and Rinderle 1988) in all sugar beet leaves. This is in agreement with observations for sunflower (Ciompi et al. 1996) and sorghum (Cechin 1998), whereas for bean (Lima et al. 1999) and wheat (Shangguan et al. 2000) a reduced efficiency of PS2 was reported when comparing between well-supplied and N-deficient plants. The $F_{690}/F_{740}$ as a non-destructive indicator of the in vivo Chl content (Lichtenthaler and Rinderle 1988, Babani and Lichtenthaler 1996) slightly decreased from low to high N-supply levels of sugar beet plants due to the increase in Chl content of the leaves. This was shown also for barley with different N-supply (Hák et al. 1993).

**Fluorescence images**: In the fluorescence imaging system FL-FIS, UV-radiation is applied in order to induce the blue-green, red, and far-red Chl fluorescence. The UV-induced fluorescence yield strongly depends on the amount of UV-absorbing, but mainly non-fluorescing soluble flavonols and cinnamic acids in the leaf epidermis. Images of the emitted fluorescence in the four spectral bands (Fig. 3A) are shown for a N12 sugar beet leaf. The blue ($F_{440}$) and green ($F_{520}$) fluorescences were predominantly emitted from the leaf veins, while the red ($F_{690}$) and far-red ($F_{740}$) Chl fluorescences were more pronounced in the intercostal leaf area. This pattern had already been observed in other dicotyledonous plants (Lang et al. 1994, 1996). Thus, the fluorescence emission was not evenly distributed across the leaf area, and a certain patchiness reflecting local differences in the actual Chl content and levels of the respective fluorophores for blue-green fluorescence emission of the leaf was detected. All fluorescence images showed a decrease in fluorescence yield along the leaf rim and at the right leaf side (Fig. 3A). The detection of gradients and local differences in fluorescence emission across the leaf area is a big advantage of fluorescence imaging (with usually more than 200 000 leaf pixels) over the hitherto applied point data measurements, containing fluorescence information of only one leaf point per measurement. The intensities of the blue-green fluorescence of the sugar beet leaves were relatively high for a dicotyledonous plant. This may be due to the fact that sugar beet leaves contain large amounts of ferulic acid (Morales et al. 1994), the main fluorophore of the blue-green fluorescence of leaves (Lichtenthaler and Schweiger 1998).

Looking at the two extremes of this study—a leaf with N-deficiency (N0) and N-luxury (N15)—the differences in N-supply can be seen in the fluorescence pattern of blue, green, red, and far-red fluorescence images (Fig. 4A). The N15 leaf also exhibited higher values for $F_{690}$ and $F_{740}$, whereas the blue fluorescence showed a smaller increase, and the green fluorescence had changed insignificantly with increasing N-content (Fig. 4A).

**Fluorescence ratio images**: The differences in fluorescence emission between the two N-supply levels can best be demonstrated by looking at the images of the

fluorescence ratios in order to eliminate external differences such as distance, geometry of the foliage, and intensification of fluorescence detection. The images of the fluorescence ratio blue/red ($F_{440}/F_{690}$) and blue/far-red ($F_{440}/F_{740}$), as early and sensitive indicators of changed growth conditions (Buschmann and Lichtenthaler 1998), allowed a clear distinction between leaf veins and other parts of the leaves (Fig. 3B). This is due, in part, to the high blue and green fluorescence emission in the leaf veins (Fig. 3A). $F_{440}/F_{690}$ and $F_{440}/F_{740}$ showed a much higher variation than other fluorescence ratios. In contrast, the images of $F_{690}/F_{740}$ and $F_{440}/F_{520}$ were homogeneous (Fig. 3B). This homogenous distribution of the ratios is achieved because they are formed by the emission of a single fluorophore each (Chl in the mesophyll cells for red and far-red fluorescence, and hydroxycinnamic acids covalently bound in the cell walls as emitters of the blue and green fluorescence). In various plants the ratio $F_{690}/F_{740}$ is an inverse indicator of the Chl content of plant leaves (Lichtenthaler and Rinderle 1988, Babani and Lichtenthaler 1996, Gitelson et al. 1999). This is based on the fact that $F_{690}$ is increasingly reabsorbed by the Chls with increase in Chl (a+b) content of leaves, whereas $F_{740}$ is little affected. For this reason the fairly homogeneous distribution of $F_{690}/F_{740}$ is an indicator for a fairly homogeneous distribution of Chl across the leaf surface.

Although the leaves analysed visually appeared homogeneously green, $F_{440}/F_{690}$ and $F_{440}/F_{740}$ of the sugar beet plant with a normal N-supply (N12) showed local differences across the leaf area (Fig. 3*B*). Significant differences were also seen between leaves of low and high N-supply plants. Both ratios declined at high N-supply (Fig. 4*B*). This is due to a lower increase in blue fluorescence as compared to the increase in the red and/or far-red Chl fluorescence from leaf N0 to N15 (Fig. 4*A*). The increase of the blue fluorescence, on the one hand, might indicate a higher accumulation of the fluorophore(s) emitting the blue fluorescence at high N, which also occurs at spots of an attack by pathogens or insects on the leaves. On the other hand, it could also be due to a lower content of photosynthetic pigments in particular leaf regions that usually re-absorb a major part of the emitted blue-green fluorescence (Buschmann and Lichtenthaler 1998). The increase of Chl fluorescence, excited by UV-radiation, with increasing Chl content (N15 as compared to N0) is due to the considerably higher concentration of Chl in the leaf mesophyll and to lower concentrations of the UV-absorbing soluble and non-fluorescing cinnamic acids and flavonols in the epidermis layer. The latter substances reduce the amount of UV radiation that passes through the epidermis into the Chl containing mesophyll cells which thus causes a lower rate of Chl fluorescence excitation and emission. Their content decreases with increasing N-supply of plants. The ratio images $F_{440}/F_{690}$ and $F_{440}/F_{740}$ were most sensitive to changed growth conditions, to plant stress, and to changes in photosynthetic activity and function (Heisel *et al.* 1996, Lang *et al.* 1996, Lichtenthaler *et al.* 1996, Schweiger *et al.* 1996, Buschmann and Lichtenthaler 1998, Subhash *et al.* 1999). They also indicate the differential N-supply as shown here for sugar beet.

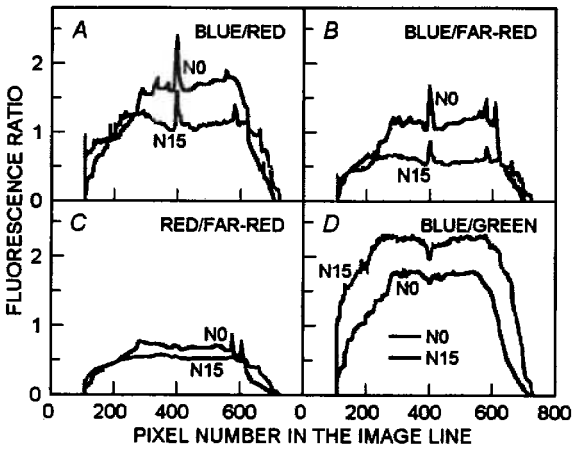


Fig. 5. Comparison of the distribution profile of the fluorescence ratios blue/red ($F_{440}/F_{690}$) (*A*), blue/far-red ($F_{440}/F_{740}$) (*B*), red/far-red ($F_{690}/F_{740}$) (*C*), and blue/green ($F_{440}/F_{520}$) (*D*) across the leaf transverse axis (horizontal profile) of a leaf from a sugar beet plant grown with no nitrogen supplied [N0 = 0 g(N) $m^{-2}$] and a leaf grown with a high amount of nitrogen supplied (N15; N-luxury) at beet harvest. The profiles are based on the fluorescence ratio images presented in Fig. 4. The profiles are based on 700 pixels per leaf.

$F_{440}/F_{690}$ of the N15 leaf was on average 1.09 and of the N0 leaf 1.61, whereas $F_{440}/F_{740}$ was 0.57 and 1.09, respectively. Thus, the blue/red ratio rose by 48 % and the blue/far-red by 91 % in the N15 leaf as compared to the N0 leaf. The increase in these ratios was mainly due to the much higher pigment content of the N15 leaf than in the N0 one (215 % for Chls and 180 % for carotenoids), not to a lower activity of photosynthesis or a decrease in electron transport. This was shown by the similarity of $R_{Fd}$ and $F_v/F_0$ in the N0 and N15 leaves (Table 2). Taking into account the ratio values of the N12 leaf from Fig. 3 (blue/red, 1.48; blue/far-red, 0.77), the N-surplus supply of 3 g(N) $m^{-2}$ in the N15 leaf decreased the fluorescence ratios by 20 % for $F_{440}/F_{690}$ and 13 % for $F_{440}/F_{740}$.

Differences in the distribution of the fluorescence ratios between N0 and N15 can be quantified by the fluorescence ratio profiles across the transversal axis of the leaf. A quantification of the differences is required to determine the statistical significance. Fluorescence imaging has the great advantage to combine high resolution (with more than 100 000 pixels per leaf) with a quantification as made possible by the profile analysis. Such horizontal profiles are presented for the two extreme N-supply levels, N0 and N15 (Figs. 5*A-D*). The Chl fluorescence ratio red/far-red (Fig. 5*C*) showed

a fairly even distribution, whereas the fluorescence ratios blue/red (Fig. 5*A*) and blue/far-red (Fig. 5*B*) were high (spikes) at the central leaf vein and at the location of the side leaf veins. The differences in fluorescence profiles of low and high N-supply showed up in all fluorescence ratios.

A further possibility of fluorescence imaging allowing to demonstrate the differences between different N-supply of sugar beet plants and to detect even small differences in fluorescence signals is the presentation of histograms of the frequency distribution of the fluorescence signals and ratios of all leaf pixels. Histograms for the fluorescence ratio blue/red and the Chl fluorescence ratio red/far-red are shown in Fig. 6.

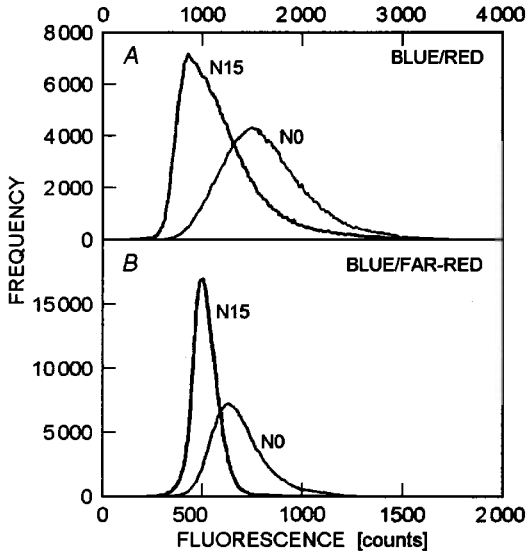


Fig. 6. Histogram of the frequency distribution of the pixel values of the fluorescence ratios blue/red (*A*) and red/far-red (*B*) in a leaf of sugar beet plants grown on zero nitrogen supply (N0) and with high N-supply (N15). The frequency distribution is based on 303 000 pixels (N15) and on 255 000 pixels (N0).

**Comparison of all N-supply sugar beet plants**: The Chl content of leaves increased with increasing N-supply in a strong linear correlation (Fig. 8*A*). As expected, $F_{690}/F_{740}$ decreased with increasing Chl content. This was seen by determination of this ratio from the red radiation induced fluorescence (Table 3) and also upon UV-excitation of Chl fluorescence (Fig. 7*B*). The absolute values for $F_{690}/F_{740}$ are higher upon red irradiation than UV excitation, but the decrease of the ratio with increasing N-supply is evident in both cases. The absolute values of $F_{690}/F_{740}$ depend on the wavelength of the excitation radiation as demonstrated by Lichtenthaler and Rinderle (1988) and Buschmann and Lichtenthaler (1998). This is due to the differential penetration depth of radiation of different wavelengths, whereby red radiation penetrates much deeper into the leaf mesophyll than blue and UV radiations.

When averaging the fluorescence ratios of all leaf pixels for the different N-supply sugar beet plant plots, $F_{440}/F_{690}$ and $F_{440}/F_{740}$ displayed most clearly the differences in N-supply (Fig. 7*B*), as already outlined for the individual leaves (Figs. 3 and 4). There is a correlation between both ratios and the Chl content of leaves as is shown for $F_{440}/F_{690}$ in Fig. 8*B*. There was little change in blue and green fluorescence with increasing N-supply, but at N12 and N15 both fluorescences increased considerably (Fig. 7*A*). Yet the promotion of the red and far-red Chl fluorescence was stronger than that of the blue fluorescence. As a consequence, a decrease of up to 40 and 35 % for $F_{440}/F_{690}$ and $F_{440}/F_{740}$, respectively, was observed in N12 and N15 as compared to N0.

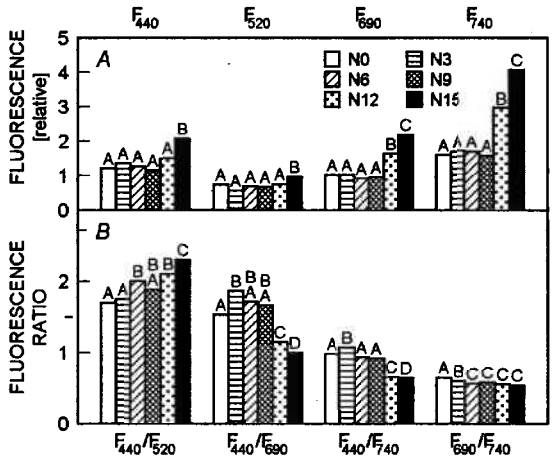


Fig. 7. Mean values of the fluorescence intensities in the four fluorescence bands blue ($F_{440}$), green ($F_{520}$), red ($F_{690}$), and far-red ($F_{740}$) (*A*), and mean values of the fluorescence ratio images for blue/green ($F_{440}/F_{520}$), blue/red ($F_{440}/F_{690}$), blue/far-red ($F_{440}/F_{740}$), and red/far-red ($F_{690}/F_{740}$) (*B*). The data were obtained from fluorescence images of the whole sugar beet leaves. The leaves were excised at harvest from N-supply plots grown with the nitrogen supplies N0, N3, N6, N9, N12, and N15 [figures give g(N) $m^{-2}$]. The columns represent the mean of seven leaves. The values are based on more than 1.5 million pixels per N-supply condition. The means with different letters are significantly different ($p < 0.05$).

The rise in blue-green fluorescence as well as in the red and far-red Chl fluorescences from N0 to N15 plants, especially in N12 and N15 (Fig. 7*A*), can be explained by a change in the optical properties and the chemical composition of leaves when grown with high N-supply. A higher transmittance of the epidermis for the UV-radiation used for fluorescence excitation under high N-supply is apparently caused by the fact that at high N-supply lower amounts of UV-absorbing soluble flavonols and cinnamic acids accumulate in the epidermis. Hence the fluorescence signatures of high as compared to low N-supply leaves resemble those of leaves from indoor plants as compared to outdoor plants as shown for maize,

oat, and wheat (Schweiger *et al.* 1996, Lichtenthaler and Schweiger 1998). The indoor plants contain much less flavonols and soluble cinnamic acids than outdoor plants (Schweiger 1999) and thus possess upon UV excitation a higher Chl fluorescence yield than outdoor plants. As a consequence, $F_{440}/F_{690}$ and $F_{440}/F_{740}$ of outdoor plants are, as here described for low N-supply, higher than in indoor plants. In the latter the ratios are similarly low as in the high N-supply leaves (Fig. 7*B*).

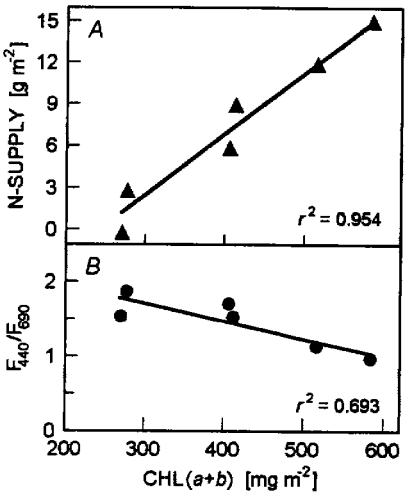


Fig. 8. Linear correlation of the N-supply and the Chl (*a*+*b*) content of sugar beet leaves (*A*) and of the mean values of the fluorescence ratio blue/red ($F_{440}/F_{690}$) and the Chl (*a*+*b*) content (*B*). The young leaves from the different N-supply plots were analysed at harvest. The fluorescence ratios are those presented in Fig. 7*B*; the Chl (*a*+*b*) values shown are the mean of 7 repetitions.

The blue-green fluorescence ratios increased steadily from 1.6 to 2.2 (35 %) from low (N0) to high (N15) N supply, based on a larger increase of the blue fluorescence as compared to the green fluorescence emission with increasing N-supply (Fig. 7*B*). This might indicate that either the concentration of a particular green fluorescing substance (*e.g.*, a flavonol, cf. Lang *et al.* 1996) decreases with increasing N-supply or an additional blue fluorophore shows up. This aspect needs further investigation. $F_{690}/F_{740}$, in turn, showed the opposite effect and dropped from 0.65 to 0.52 (18 %) at N0 to N15, indicating the increase of Chl content (Table 2). Hence the relative re-absorption of the red Chl fluorescence becomes much larger with increasing Chl content, whereas the far-red Chl fluorescence is only little affected (Buschmann and Lichtenthaler 1998, Gitelson *et al.* 1999).

*Via* the changes of all four types of fluorescence ratios of sugar beet leaves (with the exception of the blue/red and blue/far-red ratio for N0), one can detect the increasing N-supply. Multicolour fluorescence signatures of the agricultural crops wheat (Heisel *et al.* 1997), maize (Heisel *et al.* 1996), and apples (Sowinska *et al.* 1998) with different N-supply were measured with a stationary laser-FIS device. Sugar beet leaves were measured with a mobile laser-FIS for near-field remote sensing (Sowinska *et al.* 1999). All fluorescence imaging investigations proved that the fluorescence ratios blue/red and blue/far-red are the most sensitive parameters to distinguish between different N-treatments of agricultural crop plants.

**Conclusions**: Our results demonstrate that multicolour fluorescence imaging with the FL-FIS, which is based on Chl fluorescence and blue-green fluorescence, is capable to distinguish between different N-supply plots of sugar beet plants. Local differences in fluorescence intensity and fluorescence ratios across the leaf blade can be identified with a high statistical significance rendering the FL-FIS a superior technique over the previously applied point data measurements of solely Chl fluorescence. Further characterisation of the leaf health status can be achieved by evaluating the changing patterns of fluorescence signals and fluorescence ratios in a leaf image. For future routine measurements, additional calibration with standard techniques conventionally used to precisely describe the physiological state of the plants together with an appropriate automation is required. The values demonstrate that UV-induced fluorescence imaging can be applied for a non-destructive monitoring of the differences in N-supply of sugar beet plants, whereby the fluorescence ratios blue/red and blue/far-red were particularly indicative. This new method is also applicable for the remote sensing of the N-supply in the field. The Karlsruhe multicolour FL-FIS is a valuable diagnostic tool for screening site-specific differences in N-availability required for precision farming.

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