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CHANGES IN VERTICAL DISTRIBUTION OF SPECTRAL REFLECTANCE WITHIN SPRING BARLEY CANOPY AS AN INDICATOR OF NITROGEN NUTRITION, CANOPY STRUCTURE AND YIELD PARAMETERS

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The main objective of this study was to evaluate the spectral reflectance in the vertical profile of spring barley canopy at the booting growth stage and to determine how the reflectance gradient changes in relation to crop density and nitrogen (N) nutrition. Vertical gradients of spectral reflectance were studied in field trials with three sowing densities (2, 4 and 6 million of germinating seeds/ha) and two levels of N nutrition (0 and 90 kg/ha). It was found that differences in vegetation indices caused by N nutrition are most pronounced in the second and third leaf from the top, and these increase with increasing sowing density.

The vertical gradient of reflectance, specifically the ratio between the leaves F-3/F-1 for vegetation indices based on red-edge reflectance, represents a reliable indicator of number of ears per area unit (R = –0.87 for Normalised Red Edge-Red Index (NRERI) and –0.93 for Zarco-Tejada and Miller Simple Ratio Index (ZM)). A close relationship to ear productivity was found almost for all observed vegetation indices and any leaf in vertical profile (R = 0.79–0.97). In contrast, the prediction of protein content in barley grain was the most reliable when the red-edge reflectance indices (ZM and NRERI) particularly from upper three leaves were used (R = 0.81–0.88). The results show that the knowledge of reflectance heterogeneity in the vertical profile of canopy can significantly contribute to the interpretation of the measured data, to the differentiation of the N nutrition effect from the response to canopy density, and finally to a more accurate estimation of yield parameters and protein content in grain.

Key words: *Hordeum vulgare*, spectral reflectance, vertical gradient, vegetation indices, nitrogen, grain yield, protein content

Spatial variation in soil conditions and temporal changes in weather result in high spatio-temporal variability in nitrogen (N) availability with significant impact on canopy structure and crop productivity. In cropping systems, N fertilisation practices can provide a sufficient N supply for plants to achieve the potential productivity allowed by the actual climatic conditions. But because of weather variability, the applied quantities of N fertilisers are often larger than the quantity strictly required for achieving optimum yield. High weather variability combined with spatial variation in soil N supply according to soil type leads to increased risk of N leaching in most of the intensive cropping systems. Nowadays, protection of soil, water and air quality becomes a necessary constraint for agriculture, and the current fertilisation strategy is no longer sustainable.

Thus, there is high demand to improve N fertilisation strategy on the basis of N physiology knowledge and development of related remote sensing approach, which will enable to detect readily small

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changes in N status in relatively large areas. One of the most promising approaches in remote sensing is based on spectral reflectance measurements. The application of reflectance spectroscopy for the estimation of leaf pigment and N contents has recently received considerable attention (reviewed by Hatfield *et al.* 2008). Vegetation indices that combine reflectance from few spectral bands have been developed for pigment and subsequently N retrieval (Gitelson *et al.* 1996). However, the most commonly used vegetation indices such as Normalised Difference Vegetation Index (NDVI) provide a reliable estimate of the N status only if a significant deficiency is observed. Specific absorption coefficients of leaf pigments are high for blue and red wavelengths and thus even low amounts of foliar pigments are sufficient to saturate absorption (Merzlyak & Gitelson 1995). On the other hand, for the green and red-edge regions, the absorption coefficient is very low and rarely exceeds 6% of that for blue and red (Lichtenthaler 1987). Therefore, sensitivity of absorption to chlorophyll content is much higher in these spectral bands than for the blue and red spectral regions. Despite poor estimation of chlorophyll and N concentration using NDVI, good correlation to biomass and leaf area index is often reported (e.g. Alvaro *et al.* 2007).

An important limitation of remote sensing techniques is that reflectance is scanned preferentially from upper leaf layers and different vertical layers of crop canopy make different contributions to the spectral reflectance. However, the plants respond to N deficiency or other abiotic stress initially by changes in vertical allocation of nutrients and pigments aimed at optimisation of photosynthetic efficiency and prevention of upper leaves from stress impact.

The theory of optimal allocation within plant canopies predicts that nutrient concentration and photosynthetic capacity will scale linearly with gradients of light penetration (Kull 2002). However, vertical allocation of nutrients and photosynthetic activity changes during crop development (Bertheloot *et al.* 2008) and is responsive to N status (Lötscher *et al.* 2003), sowing density (Dreccer *et al.* 2000), drought (Xu & Zhou 2005) or other abiotic stresses with the aim of preventing non-shaded upper leaves from stress impact, optimising photosynthesis and assuring reproduction. Such changes in N allocation alter vertical distribution of leaf pigment concentration and senescence. Temporal changes in the vertical profile of canopy chlorophyll are driven by the distribution of chlorophyll within individual leaves that varies during the stages of its life cycle: expansion, longevity and senescence (Lizaso *et al.* 2003).

When the canopy is mature and the leaf area index is close to the maximum, the turnover of leaves and vertical gradient of N content may be close to steady state (Hikosaka 2005).

Ciganda *et al.* (2009) showed that parameters of the vertical distribution function for chlorophyll content in maize determined by spectral reflectance measurements are very useful for interpreting temporal changes in the physiological status.

Vertical gradients of leaf N optimise its utilisation with respect to carbon assimilation as an adaptation to the light gradient in dense vegetative canopies. Density and N availability affect the steepness of the N gradient relative to the photon flux density gradient and such variation in vertical gradient is related to the N status of the whole plant (Lötscher *et al.* 2003).

As the pigment composition provides basis for remote sensing observation, namely spectral reflectance (Hatfield *et al.* 2008), determination of vertical reflectance profile in combination with new spectral indices sensitive to vertical allocation may represent an important step to enhanced accuracy and timely detection of N status and to estimate individual yield parameters.

The main objective of this study was to elucidate the effect of sowing density and N nutrition on changes in the vertical distribution of spectral reflectance for more precise estimation of yield parameters. Therefore, the following hypotheses were tested: i) vertical gradient of vegetation indices increases with sowing density and N deficiency, ii) vegetation indices derived from reflectance in the green and red-edge bands improve the accuracy of N status estimation and iii) parameters of vertical distribution of spectral reflectance allow more accurate estimation of individual yield parameters.

MATERIALS AND METHODS

The field experiment on spring barley variety Prestige was conducted in 2009 in Kroměříž. The location is characterised by warm, slightly humid climate with mean annual temperature of 9.1°C and precipitation of 567.7 mm. The main vegetation season until reflectance measurements was characterised by above-average rainfall in March and below-average rainfall with higher temperatures in April. In May, the rainfall and temperatures were close to long-term average. The soil type is Luvi-haplic Chernozem and texture clay loam. The preceding crop was winter wheat. Spring barley was sown in three sowing densities of 2, 4 and 6 million of germinating seeds (MGS) per hectare. The experimental plots were fertilised with N in two doses (0 and 90 kg N/ha). The dose 90 kg N/ha was split into two separated applications: 60 kg N/ha was applied at the growth stage DC 11–12 (first to second leaf) in the form of ammonium nitrate with limestone, and 30 kg N/ha was applied by spraying of liquid fertiliser DAM 390 (mixture of ammonium nitrate and urea) at the end of tillering (DC 28–30). The plot size was 10 m^2 and the four replications were arranged in a randomised block design.

At the growth stage DC 45–49 (booting), spectral reflectance of individual leaves within vertical profile of plant (F flag leaf, F-1, F-2 and F-3 first, second and third leaf bellow the flag leaf, respectively) was measured in the range of 400–900 nm using a fibre optics spectroradiometer AVS S 2000 (Avantes, NL) equipped with a reflectance chamber using halogen light source and measuring circular area with a diameter of 1 cm. The reflectance measurements were done on 10 main stems from each replication. From a number of vegetation and chlorophyll indices we selected, based on highest response to N nutrition and sowing density, four indices covering also the reflectance in main bands related to chlorophyll content (green, red, red-edge and near infrared).

Normalised Difference Vegetation Index (Rouse *et al.* 1974):

$$\mathrm{NDVI} = (R_{780} - R_{680}) / (R_{780} + R_{680})$$

Normalised Red Edge-Red Index (Novotna *et al.* 2013):

$$\mathrm{NRERI} = (R_{780} - R_{720}) / (R_{780} - R_{680})$$

Green Normalised Difference Vegetation Index (Gitelson *et al.* 1996):

$$\mathrm{GNDVI} = (R_{780} - R_{560}) / (R_{780} + R_{560})$$

Zarco-Tejada and Miller Simple Ratio Index (Zarco-Tejada *et al.* 2001):

$$\mathrm{ZM} = R_{750} / R_{710}$$

Before the harvest, an assessment of ear number per 1 m^2 was done manually. The harvest was done using a small plot harvester Sampo 2010 equipped with automatic weighing system and sampling (Sampo Rosenlew Ltd., FI). After the harvest, a thousand grain weight was determined using grain counter Contador (Pfeuffer GmbH, DE). The grain samples were then used for analyses of the protein content (N × 6.25) using elemental analyser Leco (LECO Corporation, USA).

The basic statistical analyses (ANOVA, Tukey post-hoc test, regression and correlation analyses) were done using Statistica 8 software (StatSoft Inc., USA). Derived vegetation indices were correlated to individual yield parameters and protein content in grain.

RESULTS

Changes in vertical distribution of vegetation indices in response to N nutrition and sowing density

Vertical distribution of individual vegetation indices and its changes in relation to sowing density and N nutrition is shown in Figure 1. In general, all vegetation indices show a decrease with increasing sowing density, particularly when sowing density increases from 4 to 6 MGS. Also evident are the increasing differences in vegetation indices between N nutrition treatments with increasing density. The index NDVI shows only small response to N and also very small gradients within vertical profile. On the other hand, this index shows the highest response to the sowing density. Vegetation indices based on reflectance in the red-edge and green wavelengths (ZM, NRERI and GNDVI) show a similar pattern of response to N nutrition as well as to the sowing density. Particularly, the increase in vertical gradient with the increase in density is evident. Vertical distribution of vegetation indices is also influenced by N nutrition. Under N deficiency, the plants show a largest decline in the values of vegetation indices between leaves F and F-1. In contrast, if the N supply is sufficient, this decrease is observed only in the bottom leaves.

Correlation of vegetation indices to yield parameters

Correlation analysis of relationships between the assessed vegetation indices for each leaf within the vertical profile and yield parameters of spring barley shows that the direct yield estimation using spectral reflectance is fairly difficult and neither one of the indices showed significant correlation (Table 1). Slightly higher values of correlation coefficients were observed for the indices ZM and GNDVI particularly in F-1 and F-2 leaves. Also, the ratios between vegetation indices on leaf F-3 and F-1, which are indicators of vertical gradient of pigments within the canopy, do not contribute significantly to the improvement of yield estimation. Similarly, for the number of ears per unit area non-significant correlations to vegetation indices determined on individual leaves were observed. However, significant correlations were observed for this yield parameter and vertical gradient of vegetation indices NRERI and ZM defined as the ratio of these indices on leaves F-3 and F-1.

The results also show that the estimation of thousand grain weight using spectral reflectance is relatively difficult. The highest correlation coefficients were obtained for the red-edge index NRERI. How-

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Correlation coefficients R for individual yield parameters and vegetation indices measured in vertical canopy profile in growth stage DC 45–49

Vegetation index	Leaf				
	F	F-1	F-2	F-3	F-3/F-1
Grain yield [t/ha]					
NDVI	0.12	0.15	0.38	0.24	0.45
NRERI	0.17	0.52	0.44	0.26	-0.38
ZM	0.46	0.58	0.54	0.30	-0.63
GNDVI	0.42	0.56	0.61	0.49	0.23
Ear number [number/m^2]					
NDVI	-0.56	-0.53	-0.29	-0.45	-0.21
NRERI	-0.40	-0.13	-0.24	-0.44	**-0.87**$^{+}$
ZM	-0.25	-0.10	-0.16	-0.41	**-0.93**$^{++}$
GNDVI	-0.28	-0.10	-0.05	-0.19	-0.42
Thousand grain weight [g/1000 grains]					
NDVI	0.75	0.67	0.57	0.62	0.47
NRERI	**0.92**$^{++}$	0.62	0.67	0.69	0.56
ZM	0.62	0.42	0.46	0.52	0.46
GNDVI	0.66	0.46	0.45	0.48	0.52
Ear productivity [g/ear]					
NDVI	**0.90**$^{+}$	**0.89**$^{+}$	**0.90**$^{+}$	**0.90**$^{+}$	**0.90**$^{+}$
NRERI	0.79	**0.92**$^{++}$	**0.94**$^{++}$	**0.95**$^{++}$	0.60
ZM	**0.96**$^{++}$	**0.95**$^{++}$	**0.97**$^{++}$	**0.95**$^{++}$	0.32
GNDVI	**0.95**$^{++}$	**0.92**$^{++}$	**0.93**$^{++}$	**0.94**$^{++}$	**0.87**$^{+}$
Protein content [%]					
NDVI	0.69	0.67	0.78	0.70	0.77
NRERI	**0.82**$^{+}$	**0.88**$^{+}$	**0.85**$^{+}$	0.74	0.16
ZM	**0.88**$^{+}$	**0.82**$^{+}$	**0.81**$^{+}$	0.68	-0.13
GNDVI	**0.86**$^{+}$	**0.82**$^{+}$	**0.85**$^{+}$	0.78	0.58

F means a flag leaf; F-1 first leaf below flag leaf; F-2 second leaf below flag leaf; and F-3 third leaf below flag leaf. F-3/F-1 represents the ratio of vegetation indices between leaves F-3 and F-1. $^{++}$ indicates a highly significant correlation ($P < 0.01$) and $^{+}$ a significant correlation ($P < 0.05$). All significant correlations ($P < 0.05$) are indicated in bold.

ever, significant correlation to the thousand grains weight was observed only on the flag leaf (F). A different situation was found for the ear productivity. Statistically significant correlation coefficients were achieved through all vegetation indices, and almost for all leaf layers. On the contrary, the vertical gradient does not significantly contribute to the improvement of ear productivity estimation.

If we look at the relationships between yield parameters and selected vegetation indices that provide the best estimation (Figure 2), it is clear that a direct estimation of the yield using spectral characteristics will be affected by a significant error. A similar situation is obvious for the estimation of thousand grain weight. In this case, the best correlation was achieved for the index NRERI measured on

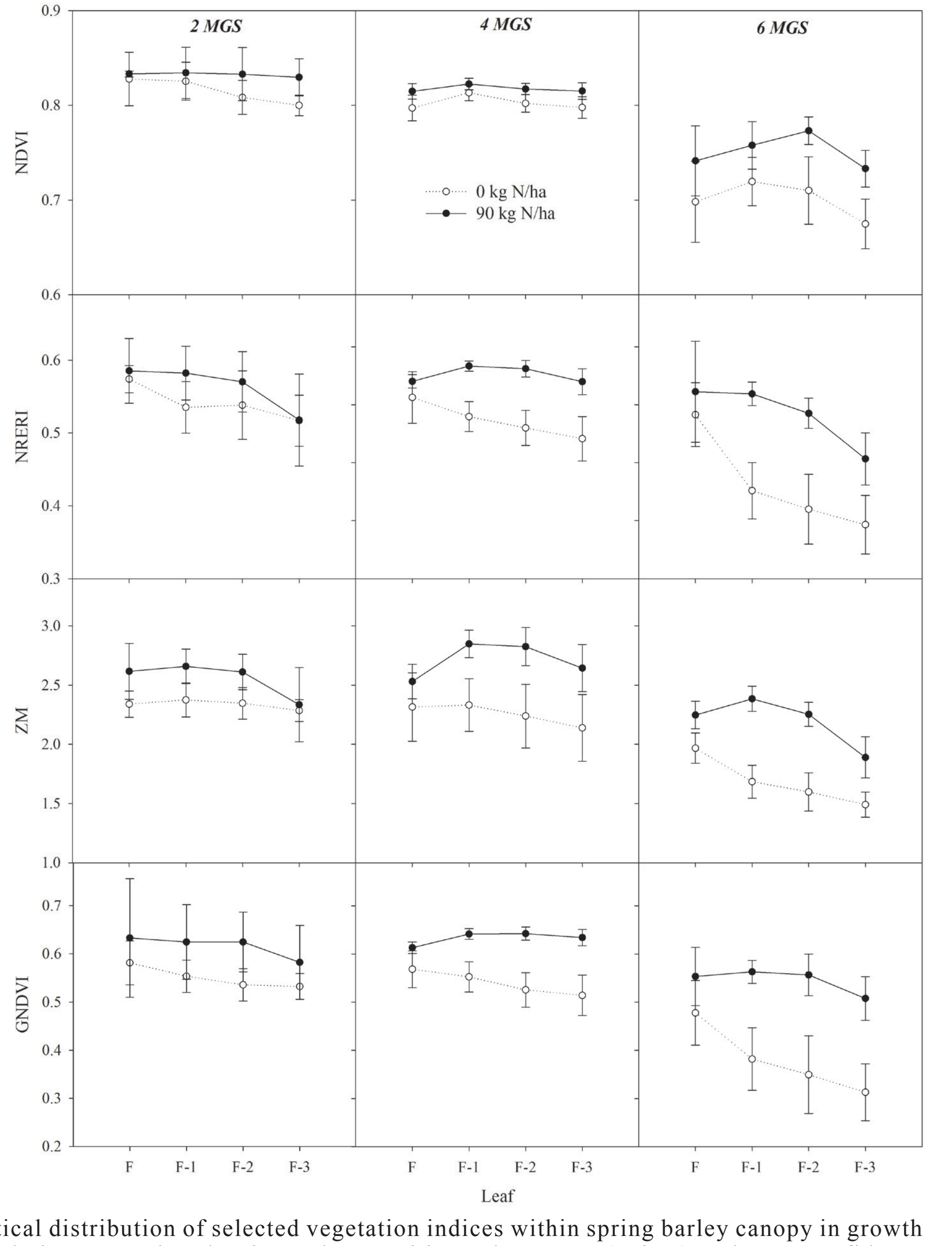

Figure 1. Vertical distribution of selected vegetation indices within spring barley canopy in growth stage DC 45–49 in relation to sowing density and N nutrition. The means (points) and 95% confidence intervals (error bars) are presented (n=4). F means a flag leaf; F-1 first leaf below flag leaf; F-2 second leaf below flag leaf; and F-3 third leaf below flag leaf. The graphs within individual columns show data for sowing densities 2, 4 and 6 MGS (million of germinating seeds).

the flag leaf. Although the statistically close relationship was found, it is necessary to take into account that the spectral characteristics on the flag leaf show high variation in general. At the same time, there is also a significant decrease of the correlation coefficients for the vegetation indices measured on the lower leaves. On the other hand, very close relationship simultaneously with relatively low variation of the vegetation indices was found for the number of ears per area unit and for the ear productivity. Number of ears is best estimated using the vertical gradient (the ratio between leaf F-3 and F-1) and the ear productivity by the red-edge vegetation index ZM determined on the second leaf under flag leaf (F-2). The combination of these two yield parameters allows for a reliable estimate of the total grain yield.

Correlation of vegetation indices to protein content

In contrast to most yield parameters, protein content in grain exhibits high values of correlation coefficients for relationships to vegetation indices, particularly in upper three leaves within the vertical profile of the barley canopy. The closest correlations were observed for the vegetation index ZM. However, a detailed study of the relationship between index NRERI and protein content in grain showed that this relationship is non-linear (Figure 3) and use of exponential function for fitting increases, for example on the leaf F-1, the correlation coefficient R from 0.88 to 0.97. This means better relationship in comparison with index ZM, which also exhibits higher variation of measured values, particularly in the flag leaf.

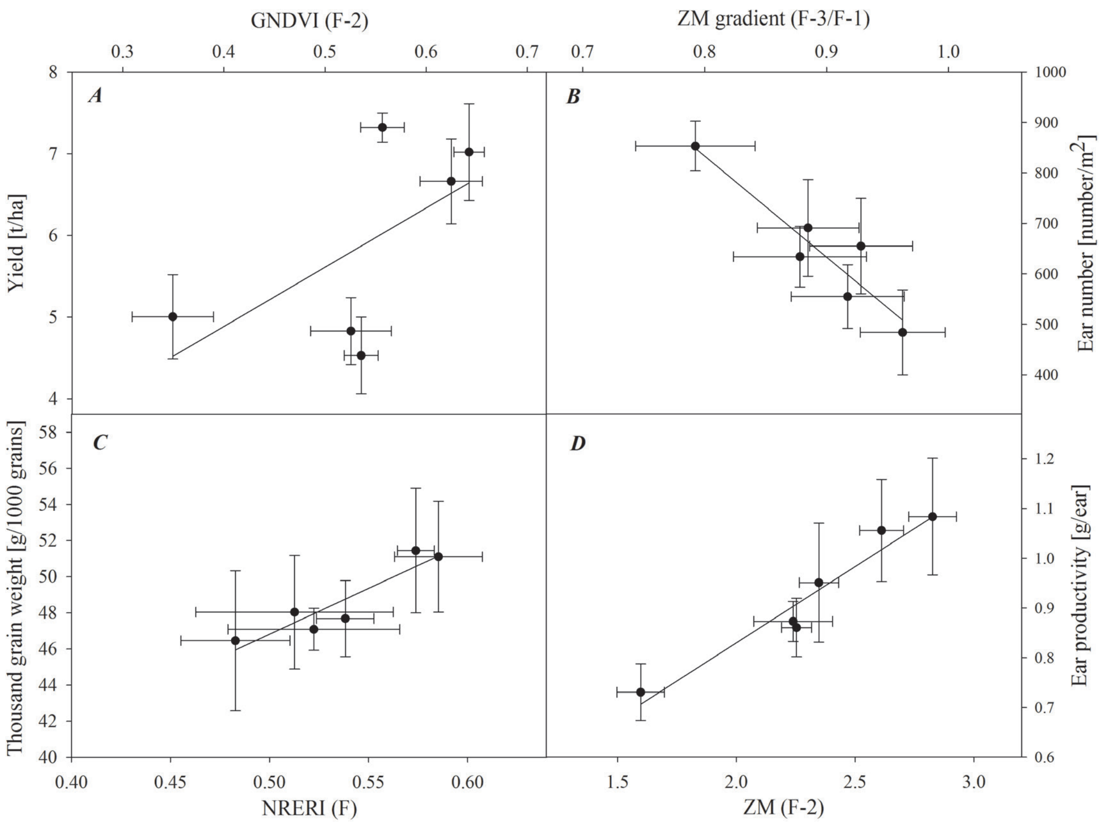

Figure 2. Relationships between individual yield parameters and selected vegetation indices/leaf position on plant (the vertical position of leaf is shown in parentheses: F flag leaf; F-2 and F-3 second and third leaves below flag leaf, respectively; F-3/F-1 ratio of vegetation indices between F-3 and F-1 leaves). The relationships with the highest correlation coefficients were selected for: A) grain yield; B) ear number per m^2; C) thousand grain weight; and D) ear productivity. The means (points) and 95% confidence intervals (vertical and horizontal error bars) are presented (n=4).

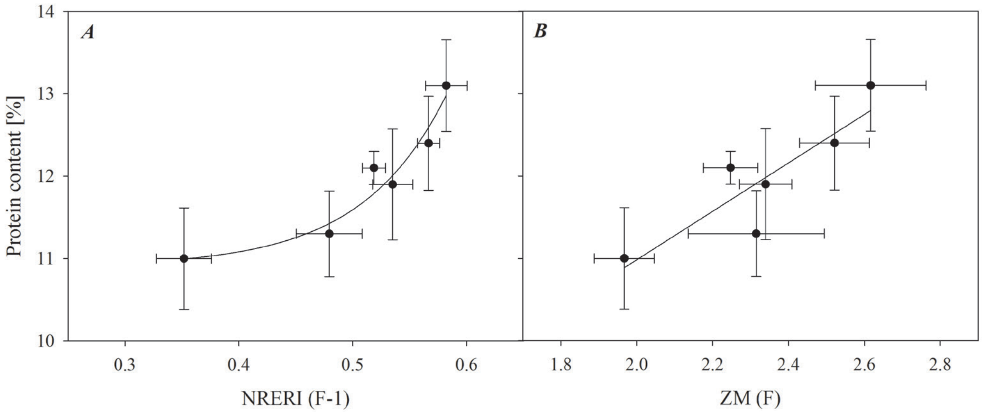

Figure 3. Relationships between protein content in barley grain and selected vegetation indices. The relationships for indices A) NRERI measured on leaf F-1 (first leaf below flag leaf) and B) ZM measured on flag leaf (F) are presented. The means (points) and 95% confidence intervals (vertical and horizontal error bars) are presented (n=4).

DISCUSSION

Spectral reflectance data function as a unique cost-effective source for providing spatially and temporally distributed information on key biophysical and biochemical parameters of vegetation. However, a fundamental problem with the reflectance sensing is the lack of generality. The shape of canopy reflectance spectra depends on a complex interaction of several factors (e.g. biochemical composition and canopy structure) that may vary significantly in time and space. Very important but often neglected part of canopy reflectance is the vertical distribution of reflectance within canopy with respect to physiological status.

As a consequence of multiple interactions of various factors, and yet insufficiently identified response of the vertical distribution of reflectance within canopy, there is no unique relationship between vegetation parameters and reflectance characteristics, but rather a family of relationships lacking the possibility to be used generally.

This is not only a problem of interpretation of the measured spectral data but also the wider applicability of vegetation reflectance models, based on radiative transfer theory, which make the assumption of a homogeneous one-layer canopy (Gobron *et al.* 1997). However, canopy generally exhibits large vertical heterogeneity of both biophysical and optical properties that has been well recognised and highlighted in recent studies (Barton 2001; Ciganda *et al.* 2008). The effects of these heterogeneities on canopy reflectance have not yet been fully addressed, to the best of our knowledge.

Our results show that the vertical distribution of reflectance is significantly changed in relation to agronomic measures. The understanding of the vertical heterogeneity sources is necessary to ensure generally valid interpretation of reflectance data and also to create a multiple-layer canopy radiative transfer model, the development of which in recent years is gaining increased attention (Wang & Li 2013).

According to our results, the vertical gradient of vegetation indices does not change in response to effects of N nutrition and sowing density as much as the shape of vertical distribution of vegetation indices. Probably due to the reallocation of N to the youngest leaves, the response of vegetation indices to N nutrition is smallest in the flag leaf. At the same time, the flag leaf reveals a high variability of vegetation indices, which is probably to the greatest extent caused by uneven time of development of the youngest leaves.

The highest effect of N nutrition on vegetation indices is evident in the second and third leaf from the top. Conversely, at the lowest leaf (F-3), a general decrease in the differences between the N nutrition treatments was observed, which may be caused by advanced senescence on this leaf layer. Similar to our results, Wang *et al.* (2005) showed a higher correlation coefficient between wheat spectral reflectance and total N content in middle leaf layers with decrease of correlation coefficients in upward direction and shift in sensitive wavelengths.

These results are different in comparison with general knowledge about vertical distribution of N within vertical canopy profile. The decline in leaf N content within the canopy tends to be steeper under low N supply (reviewed by Gastal & Lemaire 2002), which means that in the bottom leaves the differences in N content caused by N nutrition increases. This is likely due to the fact that in young leaves, which are under development and also in leaves undergoing senescence, the allocation of N to photosynthetic pigments changes and causes this disproportion between the N content and vegetation indices, which are based on changes in pigment content. The chlorophyll is during senescence degraded firstly to colourless breakdown products (NCCs) that are accumulated in the vacuole (Hörtensteiner 2006) and later reallocation of N into the newly developing leaves occurs. This means that even if the N concentration differences in bottom leaves are still high due to N availability, the breakdown of chlorophyll during senescence may cause its progressive equilibration.

An interesting interaction was observed between the effect of N nutrition and sowing density. Higher sowing density generally increases the differences between N nutrition treatments. This is probably caused by differences in light penetration within the canopy that is an important driver of vertical distribution of N and chlorophyll among leaves (Kull 2002). Vertical gradients of leaf N optimise N utilisation with respect to carbon assimilation as an adaptation to the light gradient in dense canopies. Density and N availability affect the steepness of the N gradient relative to the photon flux density gradient and such variation in vertical gradient is related to the N status of the whole plant (Lötscher *et al.* 2003). Boonman *et al.* (2007) demonstrated that accumulation of cytokinins imported through the xylem is involved in the regulation of whole-plant photosynthetic acclimation to light gradients and vertical leaf N distribution. Shaded leaves import less cytokinin than leaves exposed to high light as a result of their lower transpiration rates.

Since the variation of sowing density changes light conditions within the canopy, while also changes the vertical gradient of N and chlorophyll concentration (Dreccer *et al.* 2000), it is not surprising that the vertical gradient of vegetation indices was observed in our experiments as the best indicator of the ear number per area unit. It can be expected that, similar to the crop density, the vertical gradient will be affected by drought stress, which also accelerates senescence of older leaves and thus reallocation of N into younger leaves.

Ear productivity, which is an integral of thousand grain weight and number of grains in the ear, showed very close relationship to vegetation indices in almost all leaf layers. Generally, the most precise estimation of ear productivity was obtained using the red-edge index ZM.

Indices based on reflectance in the red-edge band seem to be a better indicator of the N and chlorophyll content in leaves, because such indices are not saturated at higher N content, which is observed for the indices based on reflectance in chlorophyll absorption maxima (e.g. NDVI; Gitelson *et al.* 1996). These results suggest that the ear productivity is closely linked with the N nutrition level.

Development of methods based on spectral reflectance to estimate the protein content in barley and wheat grain receives increased attention in recent years (Hansen *et al.* 2002; Wang *et al.* 2004; Zhao *et al.* 2005); however, the results are highly variable and hardly generally applicable. We found a very close relationship between vegetation indices based on reflectance in the red-edge band (NRERI and ZM) and also in the green wavelength (GNDVI) on the upper three leaves and protein content in barley grain. This is probably because the protein content is strongly related to N content in leaves (Wang *et al.* 2004). However, more detailed studies are necessary to clarify the complex role of sink, plant management with N and light conditions. The grains represent during reproductive stage a strong sink for N and trigger remobilisation from the vegetative organs, which decreases canopy photosynthesis and accelerates leaf senescence, depending on local light conditions and canopy structure (Bertheloot *et al.* 2008). Such complex processes may alter the relationships between spectral reflectance and protein content in a very rapid way.

CONCLUSIONS

The results of this study show that knowledge of vertical distribution of spectral reflectance within barley canopy can significantly contribute to the interpretation of remote sensing data and improve the estimation of yield parameters, particularly the number of ears per area unit. The effect of N nutrition on spectral reflectance is most pronounced in the second and third leaves from the top, and these differences increase with increasing sowing density. It can be also concluded that the use of vegetation indices based on red-edge band improves the accuracy of N effect and yield parameters estimation.

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