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Diagnosing Latent Copper Deficiency in Intact Barley Leaves (*Hordeum vulgare*, L.) Using Near Infrared Spectroscopy

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ABSTRACT: Chemometric analysis of near-infrared (NIR) spectra recorded directly on fresh leaves of barley plants (*Hordeum vulgare*, L.) enabled the separation of control and Cu deficient samples before any visual deficiency symptoms developed. This demonstrates that the molecular structure of leaves is modified during latent Cu deficiency. Lignin biosynthesis is a primary target of Cu deficiency, but lignin concentrations were unaltered when separation was first possible, indicating that alteration of lignin composition, not concentration, is among the earliest effects of Cu deficiency in plants. Validation of chemometric models using an independent test set found that 92% of samples were correctly classified as control or Cu deficient, respectively. Models were undisturbed by including spectra from plants deficient in P, Mg, B, or Mn, establishing their specificity for Cu deficiency. This study is the first to demonstrate that NIR has the potential to successfully diagnose the deficiency of an essential trace element in plants.

KEYWORDS: *barley (Hordeum vulgare, L.), copper (Cu) deficiency, NIR, spectroscopy, PCA, PLSDA, plant nutrition*

INTRODUCTION

Copper is an essential micronutrient for plants, and Cu deficiency is found worldwide, mainly in humic and sandy soils, and in soils with high pH and Cation Exchange Capacity (CEC). This results in considerable yield losses in plant production. Cu deficiency in plants can be difficult to diagnose and may not be recognized by the plant producer until it has reached a stage where correction is no longer possible. Severe Cu deficiency in cereals results in necrotic leaf tips, known as "white tip disease", which is caused by a collapse of cell walls due to poor lignification.[1] This is a key-symptom for Cu deficiency in plants, caused by a general down-regulation of the lignin biosynthesis.[2] Lignin is a biopolymer, which is partly responsible for the rigidity of plant cell walls, and it is synthesized from three phenylpropanoid alcohols: coniferyl, *p*-coumaryl, and sinapyl alcohols.[3] These monolignols are coupled to dimers and trimers by the enzyme laccase. Plant laccase is a member of the multicopper protein family, containing four Cu atoms per molecule,[4] hence the correlation to Cu status. The amount and composition of the different monolignols vary with plant species and may also change during plant development or as a result of stress.[5]

If Cu deficiency is latent, it will not result in any visual symptoms during vegetative growth. Nevertheless, plant fertility and productivity can still be severely affected either due to poor lignification of anthers resulting in failure of pollen release[6] or because the number of pollen grains is highly reduced.[7] The first visual symptom of latent Cu deficiency in cereals is, thus, a decreased grain set. This lack of clear visual symptoms in due time for action is what causes Cu deficiency to be one of the most challenging nutrient disorders to handle for the plant producer. In the case of only limited yield loss, deficiencies may never be recognized, and the prevalence of Cu deficiency could therefore be much larger than commonly accepted.

The commonly used methods for the management of Cu nutrition in crops are soil and, to some extent, plant analyses. Soil analyses are typically carried out before the start of the growing season and may thereby assist farmers in predicting fertilization needs. However, the practical use of analyzing the Cu concentration in soils has proven of little value, as the plant available concentration of a nutrient often differs significantly from the extractable nutrient concentration. Variations during a growing season may also occur, depending on changes in the soil water content.[8] Plant analyses provide total concentrations of essential nutrients in sampled plant tissue at a specific time of growth, and these are related to sufficiency threshold values. Unfortunately, the total concentration of a given nutrient does not necessarily indicate whether the plant is optimally supplied.[9] The optimal concentration of a given plant nutrient is highly dependent on plant species, cultivar, growth stage, and level of other nutrients, especially N.[1] A study in wheat and cotton found no significant differences between Cu concentrations in leaves of Cu deficient and Cu sufficient plants.[9]

In order to develop methods for simple, fast, and inexpensive plant analyses, various spectroscopic techniques have been tested for their ability to predict nutrient concentrations in plants. Near infrared (NIR) reflectance has been related to N and P status in perennial ryegrass and sugar cane, respectively.[10,11] The $L^*a^*b^*$, or CIELAB, system is a three-dimensional system using the lightness (L^*), green/red balance (a^*), and blue/yellow balance (b^*) of a color. By photographing and analyzing leaf material in accordance with this system, it has been used to assess the concentrations of N, P, K, Mg, or Fe in various legumes, *Brassica chinensis*, and maize.[12−14]

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Chlorophyll concentrations are estimated by the ratio of light transmittances at 650 nm and 940 nm in the commonly used SPAD chlorophyll meter,[15] though no strictly linear correlation to chlorophyll is found.[16] New, innovative methods count the assessment of N level in plants by combining measurements of polyphenolics and chlorophyll detected using absorbance in the ultraviolet (UV) and visual (Vis) parts of the spectrum in a hand-held device.[17] Also tractor mounted equipment is found, measuring indexes based on reflectance measurements in NIR and red light (620−700 nm). These relate to chlorophyll concentrations but not specifically to N level.[18,19] Investigations focus mainly on macronutrients, and the specificity of the methods is rarely tested thoroughly, which is absolutely critical for any practical use, as other deficiencies may occur in a field situation. Furthermore, data are related to total nutrient concentrations, which, especially for micronutrients, is not an exact measure of the nutritional status of a plant. Finally, investigations and discussions of how much growth conditions influence the results and how this can be handled are essential but yet often found missing. Altogether, this restrains the applicability of the obtained results severely.

Enabling the plant producer to carry out analyses on the farm or even in-field with results correlating directly to plant nutritional status would be a major improvement. This can be done by focusing on "the bioactive concentration", which is probed by investigating whether plant functions, for which the nutrient is essential, are hampered. Thus, the impact of any other factor influencing the optimal level is automatically taken into consideration. One successful example of such a method is the finding that Mn deficiency was correlated to the quantum yield efficiency of PhotoSystem II (PSII) in barley.[20,21]

The present work investigates the spectral differences between Cu sufficient plants and plants suffering from latent Cu deficiency by analyzing the NIR range of the electromagnetic spectrum (800−2,500 nm/12,500−4,000 cm^{-1}). The differences detected by NIR spectroscopy are related to the physiological changes induced during Cu deficiency, and it is demonstrated that the NIR technology has a potential for fast determination of the bioactive concentration of Cu in plants.

MATERIALS AND METHODS

Cultivation of Plants. Barley plants (*Hordeum vulgare* L., cv. 'Chess') were cultivated in hydroponics. In addition, plants of cv. 'Matros' were grown to produce an independent validation set. Seeds were germinated for six to eight days in vermiculite and irrigated with double demineralized water. Seedlings were transferred to black 4 L containers with nutrient solution and aerated using steel medical syringes suspended in the solution, which was changed weekly. The control nutrient solution contained 200 μM H_2PO_4, 200 μM K_2SO_4, 300 μM $MgSO_4{\cdot}7\ H_2O$, 100 μM NaCl, 300 μM $Mg(NO_3)_2{\cdot}6\ H_2O$, 900 μM $Ca(NO_3)_2{\cdot}4\ H_2O$, 600 μM KNO_3, 50 μM Fe(III)-EDTA-Na, 2.0 μM H_3BO_3, 0.8 μM $Na_2MoO_4{\cdot}2\ H_2O$, 0.7 μM $ZnCl_2$, 1.0 μM $MnCl_2{\cdot}4\ H_2O$, and 0.8 μM $CuSO_4{\cdot}5\ H_2O$. During the first week of all experiments, the concentration of micronutrients was reduced by 50% in order to avoid EDTA poisoning of the young and sensitive plants. To avoid Fe deficiency, additional 50 μM $Fe(NO_3)_3{\cdot}9\ H_2O$ was supplied this week. All stock nutrient solutions were prepared in Milli-Q water (Milli-Q Element, Millipore, MA, USA), and macronutrient stock solutions were purified by Chelex-100 resin (Sigma-Aldrich, USA) and allowed only trace impurities of cationic micronutrients. For the entire growing period, Cu was excluded from plants selected for the induction of Cu deficiency, and Mn was excluded from plants selected to develop Mn deficiency. The pH was adjusted regularly to 6.0 ± 0.3 using ultrapure NaOH and HNO_3 or HCl. Each container held 12 plants, fitted into slits in circles of rubber foam covering the top of the container. The number of containers varied in the different experiments as noted below. Plants were cultivated in a growth chamber with a light regime of 16/8 h day/night with 250−280 μmol $m^{-2}\ s^{-1}$ at plant level. Except from the time series of progressing Cu deficiency (see below), temperature was kept at 20/15 °C day/night and relative humidity at 75%.

Time Series of Progressing Cu Deficiency. A setup with 40 containers as described above was used. Twenty containers were provided with optimal, control, conditions throughout the experiment, and 20 containers were deprived of Cu throughout. Analyses were carried out daily during 10 days, from 9 to 18 days after emergence (DAE). The temperature was kept constant at 18 °C and the relative humidity at 60%.

Manganese Deficiency, Resupply of Copper, and Cultivar Variation Experiments. A setup with 60 containers as described above was used. Fifteen containers were given control conditions, 15 containers were deprived of Mn, and 30 initially deprived of Cu. The plants were measured regularly, and Mn deficient plants were harvested 31 DAE. At 32 DAE, 15 containers were resupplied with Cu and provided with control conditions for 17 days, until the end of the experiment. NIR analyses were carried out regularly throughout the 49 day growing period. In parallel with this, three containers of cv. 'Matros' provided with control conditions and three deprived of Cu were cultivated. This validation set was measured and harvested 32 DAE.

Near Infrared Absorbance Analysis. Near infrared reflectance was measured in the range from 10,000 to 4,000 cm^{-1} (1,000−2,500 nm) using a spectral resolution of 8 cm^{-1}. Data were recorded for every 3.86 cm^{-1}, resulting in a total of 1556 data points. All measurements were carried out in the middle of the youngest fully developed leaf (YFDL). The NIR spectra were recorded on a Bomem QFA Flex FT-NIR spectrometer (Q-Interline A/S, Roskilde, Denmark), but it was tested and verified that other spectrometers yielded comparable results. The measured reflectance was converted into absorbance as follows:

$$Abs = -\log\left(\frac{R}{R_0}\right)$$

where *Abs* designates absorbance, R is reflectance of the sample, and R_0 is reflectance of a white standard reference. Each leaf was mounted with the adaxial side facing the sampling window of the light source. A black object was mounted on the abaxial side to prevent any light interference. Only leaves without necrotic or chlorotic spots were measured. Measuring order of samples was randomized in order to avoid confounding treatments.

Plant Biomass and Growth Rates. Biomasses of roots and shoots of individual plants were recorded at harvest. The two parts were separated just above the seed position and weighed immediately after NIR analyses were performed. Relative growth rates (RGR) were computed as follows:

$$\mathrm{RGR} = \frac{\ln(Y) - \ln(Y_0)}{t - t_0}$$

where Y is fresh weight at measuring day t, and Y_0 is fresh weight at t_0, the first measuring day of the experiment.

Quantum Yield Efficiency. Quantum yield efficiency of PSII, expressed as *Fv/Fm*, was measured to diagnose Mn deficiency in the Mn deficiency experiment, according to the method described by Husted, et al.[20] Leaves were dark adapted for a minimum of 30 min using Hansatech leaf clips, after which the *Fv/Fm* ratios could be determined using a Handy Plant Efficiency Analyzer (Hansatech Instruments, Kings Lynn, UK). Healthy plants have *Fv/Fm* ratios around 0.83, whereas a value of 0.55 indicates strong Mn deficiency.

Chlorophyll and Carotenoid Concentrations. Concentrations of chlorophyll and carotenoids were determined in plant material from the time series experiment. Approximately 1 cm of leaf material in full width from the middle part of the YFDL was extracted for 24 h in methanol. Absorbance was subsequently measured in a Genesys 10S

UV−Vis spectrophotometer (Thermo Scientific, MA, USA), according to the method described by Lichtenthaler and Wellburn.[22]

Lignin Concentrations. Leaf material originating from plants grown in the same container was pooled in order to obtain sufficient biomass for analyses of lignin and multielemental composition. After freeze-drying, samples were ground in zirconium-coated jars containing a zirconium-coated mill ball in a Retsch MM301 ball-mill. Cell walls were isolated using the method described by Hatfield, et al.[23] in a microscaled version. Then, 10 mg samples were weighed exactly into a centrifugal filter with a 0.45 μm nylon filter (Millipore Ultrafree-MC, Millipore, MA, USA), and 500 μL of 80% ethanol was added. The samples were sonicated for 10 min at ambient temperature and centrifuged in a table-top centrifuge (Eppendorf MiniSpin, Fischer Scientific, USA) for 15 s at 14,500 rpm. This extraction step was repeated for a total of four cycles and followed by a single extraction cycle using 500 μL of chloroform/methanol (2:1). Finally, samples were rinsed twice by 500 μL of acetone and dried at 45 °C until completely dry for approximately 20 min.

The lignin concentration in cell walls was determined as described in the "microscale method using microplates" in Chang, et al.[24] Four to 6 mg of extracted cell walls were weighed exactly and transferred to 8 mL glass vials. One milliliter of 25% acetyl bromide in glacial acetic acid was added and the vials closed tightly with Teflon coated screw caps. Vials were placed in a 70 °C water bath for 30 min, shaken gently every 10 min, causing degradation of cell walls together with acetylation and bromine substitution of the lignin.[25] The digested samples were cooled on ice, and 5 mL of glacial acetic acid was added to each vial followed by vortexing. After mixing, samples were left on ice for a minimum of 30 min in order to allow residues of protein to precipitate.[26] Thirty microliters of each sample, including a blank, was transferred in triplicate to a 96-well quartz microplate. In each well, 40 μL of 1.5 M NaOH, 30 μL 0.5 M hydroxylamine hydrochloride, and 150 μL of glacial acetic acid were added, and absorbance of the lignin derivate was measured at 280 nm in a microplate spectrophotometer (Eon Microplate Spectrophotometer, BioTek Instruments, Winooski, USA). The method was verified by standard addition of 2, 4, 8, and 12% (of dry matter) pure lignin (Aldrich 471003, Sigma-Aldrich, USA) to a control sample of barley leaf. The value "absorbance per mg cell wall" was used for comparisons between samples.

Multielemental Composition of Leaves. The multielemental composition of plants was analyzed using inductively coupled plasma−mass spectrometry (ICP-MS) (Agilent 7500ce, Agilent Technologies, Manchester, UK) or ICP−optical emission spectroscopy (ICP-OES) (Optima 5300DV, PerkinElmer, Waltham, Massachusetts, USA). Prior to analysis, approximately 20 mg of each freeze-dried, ground sample was digested in 500 μL of 67−69% HNO_3 (Plasmapure, SCP Science) and 250 μL of 30% H_2O_2 (Ultrapure, Riedel de Haën, Sigma-Aldrich) using a single reaction chamber microoven digestion system (Ultrawave, Milstone S.r.l., BG, Italy). All samples were subsequently diluted to 10 mL with milli-Q water (Milli-Q Element, Millipore) and analyzed directly by ICP-MS as described by Laursen et al.,[27] or by ICP-OES as described by Laursen et al.[28] A minimum of 5 samples of digested certified reference material (spinach, NCS ZC73013, National Analysis Center for Iron and Steel, China; and apple leaves, NIST 1515, National Institute of Standards and Technology, Gaithersburg, MD, USA) was used in each analytical run for data quality evaluation. Accuracy was generally better than 90% of the reference values for all elements. Multielemental ICP-MS data was processed using the MassHunter software (version B.01.01, Agilent Technologies), while the WinLab32 software (version 3.1.0.0107, PerkinElmer) was used for ICP-OES data.

Chemometric Analyses. Chemometric analyses were carried out using Matlab R2011b (Mathworks, Inc., Natick, MA, USA) and PLS_Toolbox 6.0.1 (Eigenvector Research, Inc., Wenatchee, WA, USA). Three methods were used, namely principal component analysis (PCA), partial least squares regression (PLS), and PLS discriminant analysis (PLSDA), all explained briefly below.

Preprocessing of Spectra. Before analysis, data was preprocessed. In the present work, multiplicative scatter correction (MSC) followed by mean centering was used on all data. MSC is a standard preprocessing approach for NIR data. By correcting the individual spectra so that their slope and intercept are similar to those of the mean spectrum, the irrelevant influence of scatter and offset is minimized.[29] Mean centering is done by subtracting the mean of all spectra included in the model from each individual spectrum in order to focus on the variation between samples.

Principal Component Analysis. Principal component analysis is a method for reducing the number of dimensions in multivariate data with a minor loss of information, thereby enabling a simpler, yet comprehensive, overview of the main variations within the data set. Data are visualized on information-rich axes named principal components, where often the first few will be sufficient for showing major differences between samples. For a more thorough introduction to PCA, see Martens and Næs.[30]

Partial Least Squares and Partial Least Squares Discriminant Analysis. The partial least-squares analysis has many similarities to PCA, only where the new axes, here named latent variables, are determined so as to maximize how much they are able to covary with a set of responses in a *y*-matrix, for instance total concentrations of a nutrient. The outcome is a regression model which is able to predict *y*-values based on *x*-input. The PLSDA is an extension of the PLS analysis, yielding a model focused on finding the variation that separates two or more groups. Barker and Rayens[31] discuss the method more thoroughly.

Validation and Cross-Validation. When the number of samples does not allow a separate validation set, as in most of the present work, cross-validation is used instead. Cross-validating a model means that a number of models are computed, excluding in turn all data in groups.[32] The error of the predictions of *y* of the excluded data provides an estimate of the error that would be obtained when predicting truly new samples with the model. In the present work, plant samples grown in the same container were excluded groupwise in the cross-validation, so that the number of cross-validation groups equaled the number of containers.

A PLSDA model was developed using 982 samples of cv. 'Chess' pooled from different, preliminary experiments. The experiments were carried out under a variation of growing conditions, in both growth chambers and the greenhouse, and P, Mg, Mn, and B deficient plants were included in the control group. The model was validated on a validation set containing 72 samples (cultivated in 6 containers) of cv. 'Matros'. The independence of the validation set can be disputed by the growing conditions, which were similar for the validation set and 120 samples of the calibration set. It should be noted, though, that the cultivars and measuring days differed.

RESULTS

Plant Growth and Development of Cu Deficiency Symptoms. The first visual difference between Cu treatments in all experiments was a retardation of both shoot and root growth in plants deprived of Cu, which increased clearly with time (Figure 1). No Cu deficiency symptoms were apparent on the leaves during the experimental period, but the characteristic "white tip disease" developed in plants deprived of Cu when they were cultivated for an extended period of up to 49 DAE (Figure 2).

Chlorophyll and Carotenoids. A significant ($p < 0.0001$) elevation of average carotenoid concentrations in plants deprived of Cu, compared to that in control plants, was shown throughout the time series (Table 1), indicating that the plants were stressed. No differences were found in chlorophyll concentrations between treatments (data not shown).

Elemental Composition of Plants. Multielemental analysis showed that Cu was the only essential plant nutrient differing consistently in concentrations between treatments. In control plants, a slightly declining trend in the high Cu concentrations was noticed from 10 DAE and onward (Table 2). Similar observations were made in control plants of the

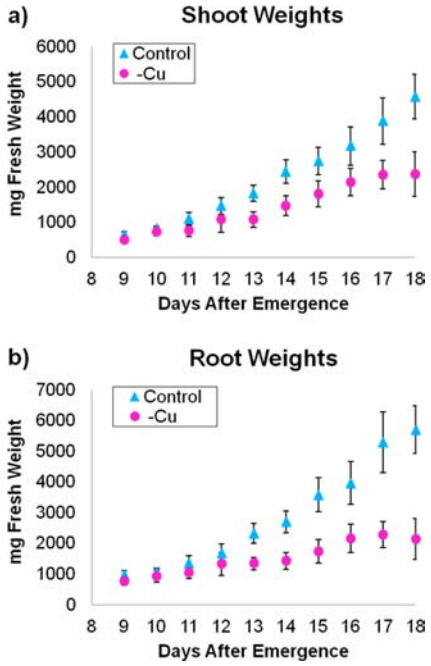

Figure 1. Shoot (a) and root (b) fresh weight of control and increasingly Cu deficient plants from the time series of progressing Cu deficiency. Values are shown as means (n = 24) ± 1 standard deviation (SD). Means of treatments were significantly different in both shoots and roots according to Student's t test during the entire measuring period.

Figure 2. Youngest fully developed leaf of control and heavily Cu deficient plants at 49 DAE. Healthy leaf from a control plant (top) and a leaf with clearly developed Cu deficiency symptoms, "white tip", from a plant deprived of Cu (bottom).

Table 1. Total Concentrations of Carotenoids (mg/g Fresh Weight) at 9-13 DAE in Control and Cu Deficient Plants from the Time Series of Progressing Cu Deficiency[a]

	treatment	
DAE	control	−Cu
9	0.06 ± 0.04	0.11 ± 0.06
10	0.05 ± 0.02	0.10 ± 0.03
11	0.04 ± 0.03	0.15 ± 0.04
12	0.07 ± 0.03	0.11 ± 0.08
13	0.15 ± 0.04	0.20 ± 0.05

[a]Values are shown as means (n = 24) ± 1 SD. Concentrations differed significantly according to Cu treatment (p < 0.0001).

experiment running until 49 DAE. Plants deprived of Cu for the entire period contained little Cu, whereas Cu deprived plants resupplied with Cu at 32 DAE increased their Cu concentrations rapidly and even exceeded the level in control plants slightly but at a significant level (Table 3).

Lignin Concentrations. Lignin concentrations were assessed in the time series of progressing Cu deficiency. Comparing control samples with those deprived of Cu, no

Table 2. Total Cu Concentrations (μg g^{-1} Dry Weight) at 9-13 DAE in Control and Cu Deficient Plants from the Time Series of Progressing Cu Deficiency[a]

	treatment	
DAE	control	−Cu
9	10.1 ± 0.2	2.4 ± 0.0
10	15.2 ± 0.6	2.2 ± 0.0
11	14.7 ± 0.4	1.6 ± 0.1
12	13.0 ± 0.2	2.5 ± 0.0
13	13.2 ± 0.1	1.8 ± 0.0

[a]Values are shown as means (n = 2) ± 1 SD. All measuring days and means of treatments were significantly different according to a Student's t-test.

Table 3. Total Cu Concentrations (μg g^{-1} Dry Weight) in Control, Cu Deficient, and Cu Resupplied Plants from the Cu Resupply Experiment[a]

	treatment			
DAE	control	−Cu	Cu resupplied	n
28	20.3 ± 4.9	0.9 ± 1.1		5
35	17.1 ± 2.1	<LOD	4.6 ± 1.5	6
44	10.7 ± 2.0	<LOD	13.4 ± 1.4	3
49	11.6 ± 1.2	<LOD	14.6 ± 1.5	5

[a]Measurements are derived from 28-49 DAE, where 35-49 DAE is equivalent to 3−17 days after resupplying Cu. Values are shown as means (n indicated in table) ± 1 SD, and concentrations below the limit of detection (LOD) are designated <LOD. All measuring days and means of treatments were significantly different according to a Student's t-test.

significant differences were observed until 14 DAE. After that time, lignin concentrations in Cu deficient plants were lower compared to those of control plants, though an overall increasing tendency was noted for concentrations in samples of both treatments (Figure 3). At 17 DAE, the samples had

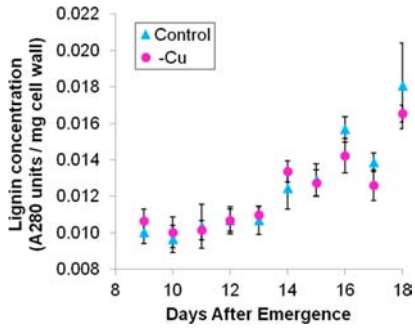

Figure 3. Lignin concentrations in the YFDL of control and increasingly Cu deficient plants from the time series of progressing Cu deficiency. Expressed in units of absorbance at 280 nm per mg isolated cell wall material after derivatization of lignin. Values are shown as means (n = 2) ± 1 SD. Means of treatments were significantly different from 14 DAE according to a Student's t test.

somewhat lower concentrations than expected and must be regarded as outliers. This is not an unexpected incident, as the number of biological repeats is very low.

NIR Analysis. NIR absorbance spectra were measured directly on the adaxial surface of the YFDL of all plants. Copper has restricted phloem mobility in plants, which is why the expression of deficiency is expected first in the youngest leaves.

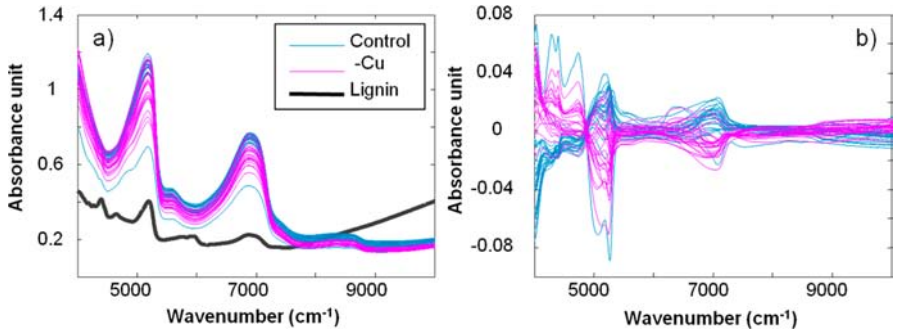

Figure 4. Raw (a) and preprocessed (b) NIR spectra from 13 DAE in the time series of progressing Cu deficiency; the spectrum of pure lignin is inserted in panel a. Preprocessing was carried out using MSC and mean centering.

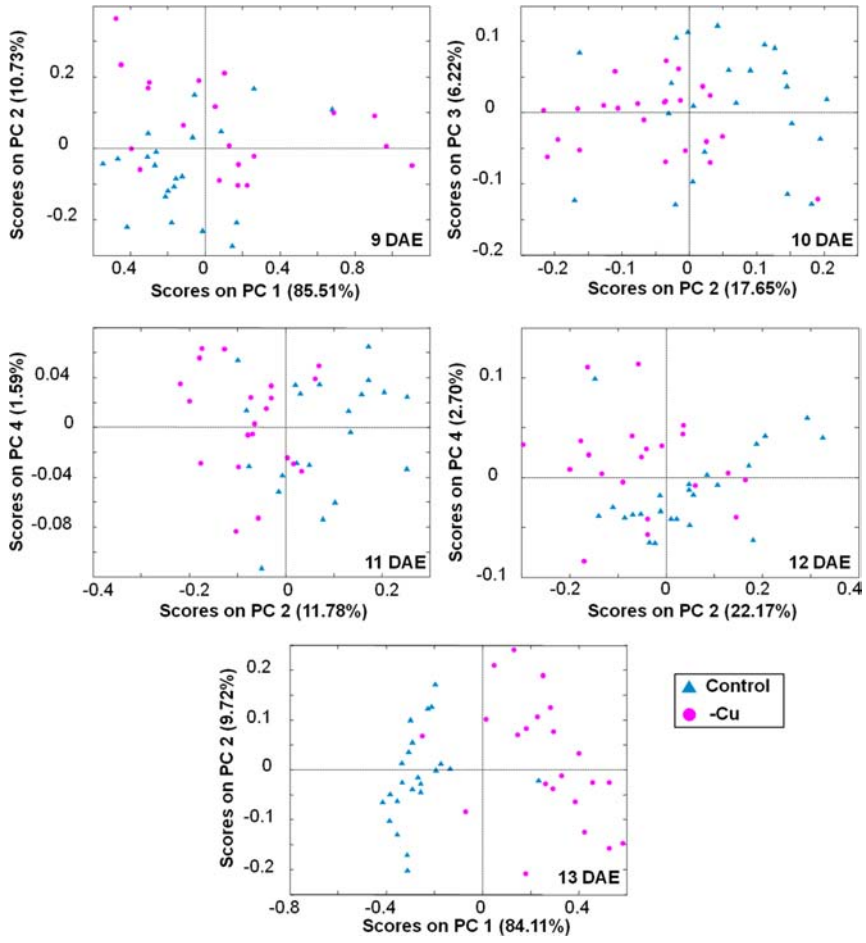

Figure 5. Score plots for PCA models based on NIR spectra from plants in the time series of progressing Cu deficiency. The principal components performing optimal separation of treatments in each model are presented, from 9−13 DAE.

The adaxial side was chosen due to practical considerations, as the leaf was the easiest to place on the NIR instrument this way. In Figure 4, an example of raw and preprocessed spectra is shown together with the raw spectrum of pure lignin. Using only raw spectra, no immediate classification of an unknown spectrum would be possible due to the overlapping of spectra from the two treatments. After preprocessing, the spectra separate systematically according to treatments in the beginning of the spectrum, at 5,200−5,300 cm^{-1} and at 6,800−7,100 cm^{-1}. At these specific ranges, the spectrum of lignin is also found to peak. From 7,500−10,000 cm^{-1}, the preprocessed spectra are similar for both treatments and contain little information about the plant tissue chemical composition. This was found to be a general pattern for all spectra measured, and consequently, this part of the spectrum was omitted before modeling.

Partial Least Squares Model. We tested whether the obtained NIR spectra could be related to total Cu concentrations in leaves, using a PLS model developed on data from the Cu resupply experiment. The Cu concentrations in leaf tissue span the range from below the limit of detection and up to almost 28 μg Cu g^{-1} dry matter. A calibration based on 7 latent variables resulted in a cross-validated model with a root mean squared error of cross-validation (RMSECV) of 5.7 μg Cu g^{-1} dry matter. The RMSECV is the average error in cross-validation and hence a measure of the inaccuracy of the

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predictions. With a concentration range from below the detection limit to 28 μg Cu g^{-1} dry matter, 5.7 μg Cu g^{-1} dry matter must be considered a relatively large error value. The poor prediction quality of the calibration is further substantiated by the squared correlation coefficient (R^2) for cross-validated data, which is 0.5. Thus, a model based on NIR absorbance data is unlikely to predict the total concentration of Cu in leaves at a satisfactory level.

Principal Component Analysis Models. In the time series of progressing Cu deficiency, spectral data were analyzed for each separate measuring day by PCA using 2 to 4 principal components and investigating the ability of the model to separate samples into two groups according to Cu treatment. The separation between treatments improved daily from 9 DAE, when the first analyses were carried out, to 13 DAE, when an almost complete separation was obtained (Figure 5). From 14 to 18 DAE, the separation of groups remained close to complete, with only few outliers as exceptions (data not shown). The loadings of the first principal components were found to peak in the same ranges as the raw spectra, i.e., 5,200−5,300 cm^{-1} and 6,800−7,100 cm^{-1} (loadings not shown). Hence, depriving plants of Cu affects molecules with strong absorption in these specific ranges, thereby enabling a separation of Cu treatments using the spectral information.

Analyzing all NIR spectra collected during the time series of progressing Cu deficiency in one PCA resulted in a model exhibiting a clear effect of age (Figure 6a). The first principal component declines in value with increasing sample number, i.e., increasing age of the plant. In the second principal component, the effect of Cu treatment is found. Samples are separated according to treatment from sample number 234, equivalent to 14 DAE, and onward (Figure 6b). This is one day later than the first occurrence of complete separation by a model based on data from only one day at a time (Figure 5).

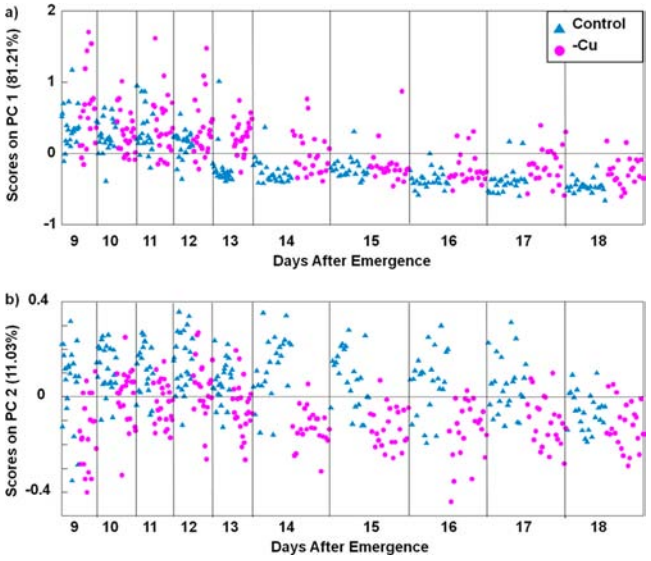

Figure 6. Score plots presenting the first (a) and second (b) principal component versus measuring day (DAE) of a PCA model based on all NIR spectra collected in the time series of progressing Cu deficiency. One batch of control and Cu deficient samples was measured each day. The order of samples within the measuring day and treatment is random. The first and second principal components correspond predominantly to the effect of age and Cu treatment, respectively.

Validation of Specificity. The physiological effects of Mn and Cu deficiencies have a number of similarities, and they are therefore likely to be confused. Hence, the specificity of the method using NIR absorbance spectra to detect Cu deficiency could be examined to some extent by investigating whether Mn and Cu deficiencies differ in spectral fingerprints. This was tested in a setup where both disorders were induced, along with the cultivation of control plants with ample supply of nutrients.

Severe but still latent Mn deficiency was established 31 DAE, around Zadoks growth stage 23, with *Fv*/*Fm* ratios of 0.55. Control plants had *Fv*/*Fm* ratios of 0.82, and Cu deficient plants were only slightly lower, at 0.79, which does not indicate any critical PSII malfunctioning of the plant. The NIR absorbance spectra of the YFDL on all plants were measured on this day and a cross-validated PCA developed. The score plot of this model shows that Cu and Mn deficient plants have separated from control plants and from each other along the first and to some extent the second principal component (Figure 7).

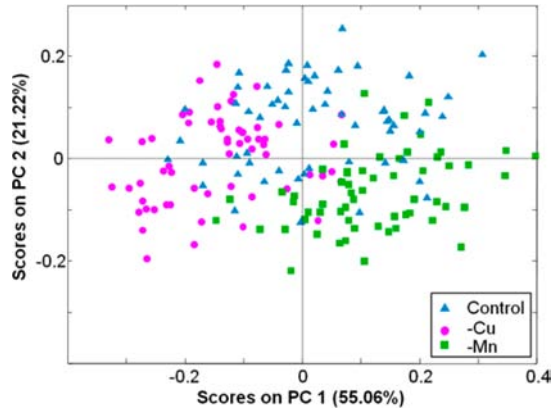

Figure 7. Score plot of a PCA model based on NIR spectra from control, Cu deficient, and Mn deficient plants 31 DAE. The two deficiencies separate mainly along the first principal component, whereas especially control plants tend to have higher values along the second principal component.

A method for diagnosing a nutritional disorder must ideally be efficient at a stage where the disorder can be remedied and the plants brought back into a growth condition similar to that of control plants. Whether models based on NIR spectra fulfill this requirement was tested by resupplying Cu to plants suffering from latent Cu deficiency as indicated by comparing NIR absorbance spectra with those of control plants. Thirty-two DAE, at Zadoks growth stage 22−23, Cu deficiency was clearly established according to a cross-validated PLSDA model with four latent variables on the NIR absorbance spectra of the YFDL (Figure 8), and half of the Cu deficient plants were resupplied with Cu to the same level as that used in the control treatment. The subsequent plant response was followed by measuring NIR spectra regularly in order to establish whether the deficient plants resupplied with Cu were brought back into a healthy state. Each measuring day, a PLSDA model was constructed based on control and Cu deficient samples. Using this model, the Cu resupplied plants were predicted to see when the major part would be classified as control plants. Four of the PLSDA models are shown in Figure 9, based on data from 3, 10, 14, and 17 days after resupply (DAR) or 35, 42, 46, and 49 DAE (Zadoks growth scale 23−29), and they used 6, 6,

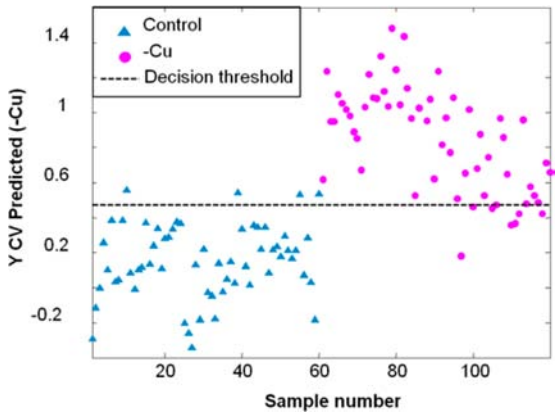

Figure 8. Cross-validated predictions of Cu treatment according to a PLSDA model based on NIR spectra from control and Cu deficient samples measured 32 DAE. Four latent variables were used in the model. The dashed line indicates the optimal separation of treatments, resulting in a clear division.

7, and 9 latent variables, respectively. It is seen that the spectra of the YFDL of the Cu resupplied plants gradually became comparable to those of the control plants.

NIR spectra of 982 samples from different, preliminary experiments were pooled in a common calibration set, and a PLSDA model separating control from Cu deficient samples was developed. Plants deficient in P, Mg, Mn, and B alone or in combination with Cu deficiency were included in the calibration set, according to Cu status, in order to maximize robustness. The model was validated on a 72 sample validation set, half of which were control samples, and half were deprived of Cu. In order to introduce variation between cultivars in the investigation, these plants were of cv. 'Matros'. Sixty-six of the 72 validation samples, or 92%, were classified correctly using the developed PLSDA model.

A model developed exclusively on 120 calibration samples, out of the 982, which were cultivated under similar growing conditions as those of the validation set, was able to classify 62 out of 72, or 86%, of the validation samples correctly. Hence, including only samples cultivated under similar growing conditions in the calibration and validation sets did not improve the performance of the model. Leaving out the same 120 samples of the calibration resulted in a model that classified the validation set as outliers, thereby showing that growing conditions are of major importance to the NIR spectra of barley leaves.

DISCUSSION

Score plots of PCA models show that it is possible to distinguish between control and Cu deficient leaf samples based on NIR spectra (Figure 5). As Cu deficiency progressed, the separation improved, demonstrating that the molecular structure of plants exposed to different Cu treatments differed more and more from control plants. The only visual symptom present was growth retardation of roots and shoots when compared to those of control samples. In a field situation where

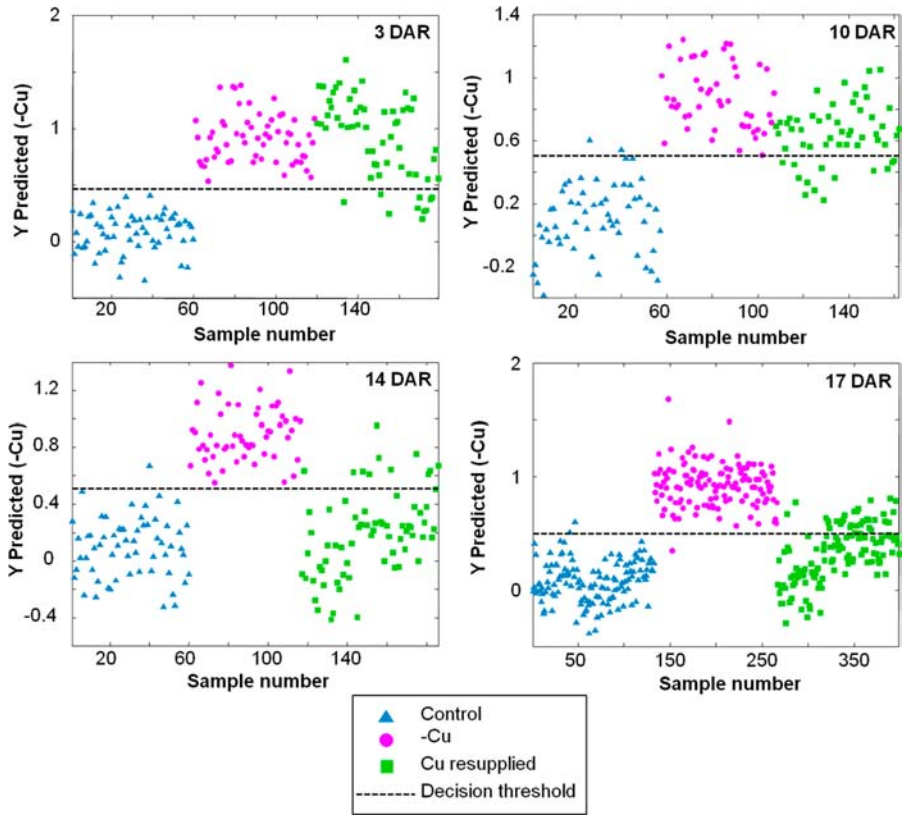

Figure 9. Predictions of Cu resupplied plants according to PLSDA models based on NIR spectra from control and Cu deficient plants. *X*-axes show sample numbers, i.e., they refer to the treatment of the samples. The dashed lines indicate the optimal separation of treatments. Data are derived from 3, 10, 14, and 17 days after resupplying Cu. The models use 6, 6, 7, and 9 latent variables. With time, an increasing part of the Cu resupplied samples are predicted as control plants. From 14 DAR, they are predominantly predicted as control samples, and this picture has not changed 17 DAR.

no control plants are available, the deficiency would therefore be visually undetectable.

Validity of Method. The specificity of the method was tested in a setup where both Cu and Mn deficiencies were induced, along with the cultivation of healthy control plants. Copper and Mn deficiencies to some extent affect the same processes in plants, which is why Mn deficiency is the disorder most likely to be mistaken for Cu deficiency using NIR. Both micronutrients are essential to the biosynthesis of lignin, which is downregulated during deficiency,[1] and both are components of SOD's, i.e., Mn-SOD and Cu−Zn−SOD. Also, photosynthesis is affected by the deficiency of both Mn and Cu, with Mn deficiency mainly depressing PSII[20] and Cu deficiency mainly affecting the activity of PSI.[1] A PCA model successfully separated NIR spectra of Cu and Mn deficient samples from both control plants as well as each other, before any visual deficiency symptoms had appeared (Figure 7). Half of the Cu deficient plants were resupplied with Cu when clearly separable from control plants using PLSDA on NIR spectra (Figure 8). Fourteen days after resupply, the resupplied plants were predicted, according to a PLSDA model, as belonging to the control group or just around the threshold value separating treatment groups (Figure 9). Hence, it is shown that a hidden Cu deficiency, detectable using NIR spectra, can be remedied and appears not to have caused any irreversible damage at this stage of development. Cu concentrations in the resupplied plants rapidly rose to levels significantly higher than those of control plants (Table 3). It is, thus, demonstrated that plant metabolism, as reflected by the NIR spectra, needs time to equilibrate after having been exposed to a nutrient disorder, though a sufficient amount of nutrient is provided rapidly.

Further testing of the specificity was carried out by using PLSDA models, which are focused on separating groups and which produce actual predictions. A PLSDA model was developed, based on 982 samples pooled from various experiments carried out in different climatic conditions, harvested at different ages and levels of Cu deficiency, and including plants subjected to other nutritional disorders (Mn, Mg, B, and P) in addition to Cu. This model was able to predict 92% of a 72 sample validation set of a different cultivar, 'Matros', correctly. Limiting the calibration set to 120 samples, which were cultivated under the exact same growing conditions as the validation set, decreased the correct classifications slightly to 86%. This shows that the advantage of similar growing conditions and ages for calibration and validation sets can be outweighed or at least balanced by including a large number of samples covering a range of growing conditions, even combined with several other nutritional disorders and across various ages in the calibration set. Thus, it is demonstrated clearly that the chemometric models developed from NIR spectra were able to identify general characteristics in the spectra, which are indicative of Cu deficiency, even when applying models to a different cultivar. When the 120 samples were excluded from the calibration set, the validation set became an outlier to the model, showing that growing conditions do affect the spectral characteristics to a high degree. In order to develop a robust model that can be used in practical agriculture, it will therefore be necessary to collect a wide variety of data from plants grown under different climatic conditions, in a number of seasons, and probably also from as many different geographical locations as possible. Though genotype in this case appears to be of little importance, most likely numerous cultivars must be included to develop a generally applicable model.

Growth Characteristics. Lignin concentrations in leaves of control and Cu deficient plants in the time series of progressing Cu deficiency did not differ significantly until 14 DAE (Figure 3), the day after complete separation was found using a PCA model (Figure 5). This result was surprising, as lignin concentration in leaves was previously found to decrease during Cu deficiency,[33] albeit this finding was derived from considerably older plants than those in the present experiments. The spectrum of pure lignin (Figure 4) in the range from 4,000 to 7,500 cm^{-1} peaked at the same wavelengths as the loadings of models separating control from Cu deficient samples, indicating that lignin could be responsible for the separation of groups. Curiously, when beginning separation between Cu treatments was noted in PCA score plots, no separation was found for lignin concentrations yet, which is why this might not be the sole factor causing separation of Cu treatments. During stress, the organization and chemical composition of lignin in the cell walls have previously been shown to change, as observed in black cottonwood using FT-IR.[5] NIR spectra are the overtones of signals observed in the IR part of the electromagnetic spectrum, why similar changes are very likely to be detectable also using NIR. Specifically identifying the lignin structures in a new investigation would give more clarity regarding the degree of organizational change and how fast it occurs.

The presence of latent Cu deficiency in plants deprived of Cu was stated in all experiments by the gradual decrease in total Cu concentrations, accompanied by stunting of growth (Tables 2−3 and Figure 1). We observed that only when cultivating plants deprived of Cu for a prolonged period, up to 49 DAE, serious "white tip disease" developed. Supporting the stressed state of the Cu deficient plants is the increase in carotenoids concentrations relative to control plants, as these generally rise when the photosystems or photoprotective components are damaged as during Cu deficiency.[1,34]

Perspectives for Practical Use. Commercial databases for NIR-based grain analysis have been developed by several private companies, including FOSS Analytical, which has been pioneering the application of NIR and Fourier transform−infrared (FT-IR) analysis on food and agricultural products. At first, predictions were only reliable for samples within a very limited geographical origin, but stability of the calibrations increased steeply with increasing numbers of growing seasons and locations included. After collecting more than 30,000 samples from all over the world during 25 years, these calibrations now span a huge variation, and an accuracy superior to routine wet chemistry on common samples has been obtained.[35,36] Likewise, a very large data collection may be necessary in order to build a global calibration for the detection of Cu deficiency in barley. Local calibrations may be developed using much smaller data sets, but for robustness, a number of seasons should be included since climate and other growing conditions are, as shown, factors of great influence on the spectra of barley leaves.

The phenomenon of increasing quality of calibrations with increasing variation and size of the data set has also been described by Xu, et al.,[37] who developed PLS models based on NIR spectra to predict concentrations of chlorogenic acid in plant extracts during ethanol precipitation of starch, protein, polysaccharides, and inorganic acid salts. For predicting N concentration in grasses using NIR, it has been shown that the effect of year of growth is of significant influence, and a number of years have to be included to develop a robust calibration.[11]

On the basis of this, it is suggested that by careful and broad selection of calibration samples, a robust calibration for detection of Cu deficiency in cereals may be developed even though a massive database is not yet available.

An attempt to develop a PLS model based on NIR spectra to predict Cu concentrations in plants resulted in very poor performance. This is in full accordance with previous findings for Cu and other essential nutrients, even when combining NIR with Vis spectra,[38] and is explained by the fact that spectra do not contain direct information about concentrations of elements but are affected by molecular movements.[39,40]

Cu deficiency is not only a problem limited to cereal production. In plantations of pine and eucalyptus species, Cu deficiency comprises a limitation to optimal growth and development.[41,42] Similar problems are also observed in the horticultural plant production, including the cultivation of Prunus species.[43] Thus, it would be of interest to investigate whether NIR analyses of dicot leaves also show a specific correlation to Cu deficiency and sufficiency and, if they do, develop a method for diagnosing disorders at early stages also in these species.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

Abs, absorbance; DAE, days after emergence; DAR, days after resupply; FT-IR, Fourier transform infrared; ICP-MS/-OES, inductively coupled plasma−mass spectrometry/optical emission spectroscopy; LOD, limit of detection; MSC, multiplicative scatter correction; NIR, near infrared; PCA, principal component analysis; PLS, partial least squares; PLSDA, partial least squares discriminant analysis; PSI/PSII, photosystem I/photosystem II; R^2, squared correlation coefficient; RGR, relative growth rate; RMSECV, root mean squared error of cross-validation; SD, standard deviation; SOD, super oxide dismutase; UV, ultraviolet; Vis, visual; YFDL, youngest fully developed leaf

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