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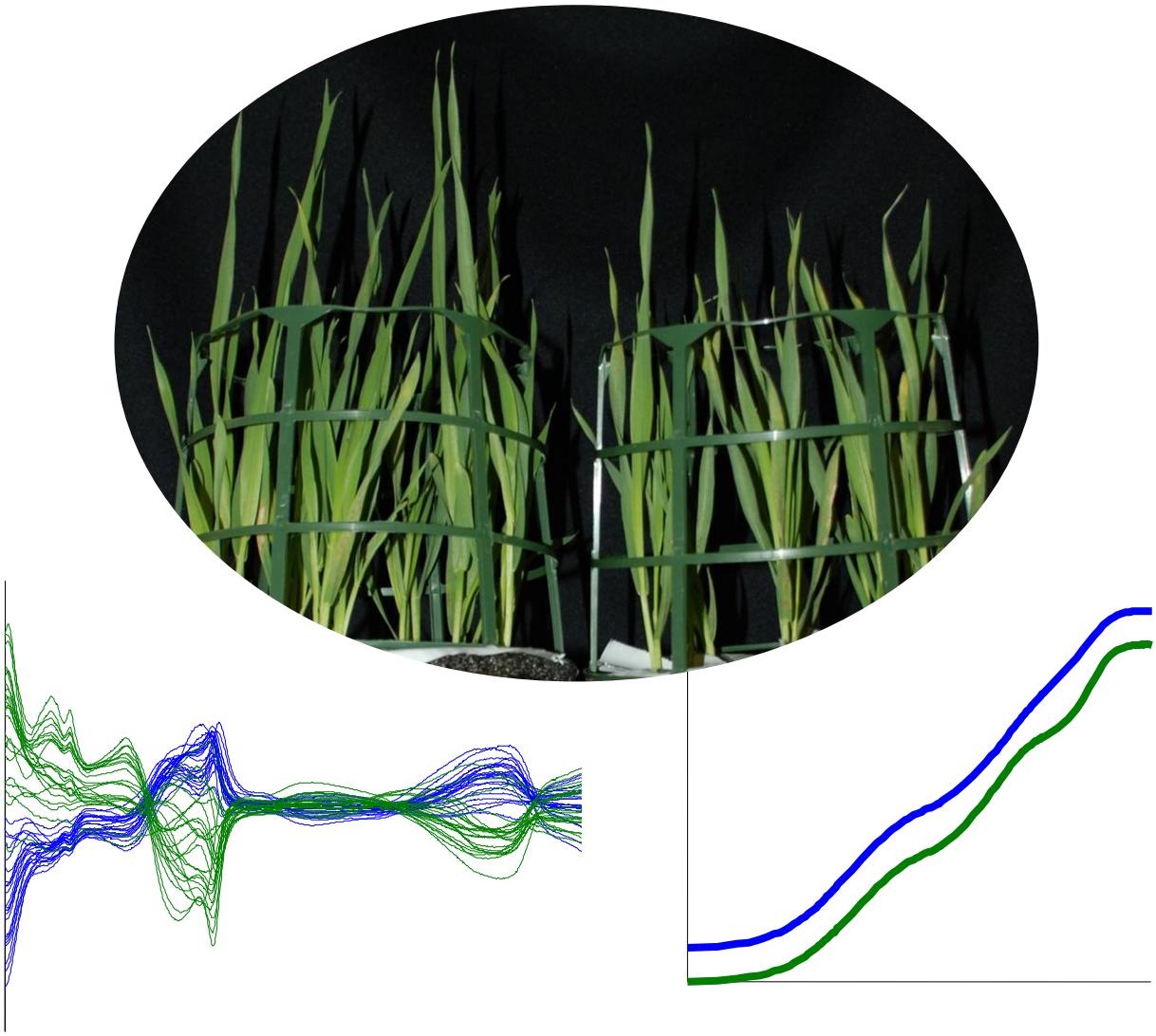
New Ways to Determine Plant Nutrient Deficiencies Using Fast Spectroscopy



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Industrial PhD Thesis

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New Ways to Determine Plant Nutrient Deficiencies Using Fast Spectroscopy

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Cover illustrations

Healthy barley plants alongside barley plants with latent Cu deficiency. Preprocessed NIR spectra from healthy and Cu deficient barley plants. OJIP transients from a healthy and a P deficient barley plant.

Preface

This PhD thesis is an industrial PhD thesis carried out as a collaboration between FOSS Analytical A/S and two research groups at Faculty of Science, University of Copenhagen: Plant Nutrition, Department of Plant and Environmental Sciences, and Quality and Technology, Department of Food Science. The project was granted by the Danish Agency of Science, Technology and Innovation and FOSS Analytical A/S.

The project was supervised throughout by Professor Søren Husted and Professor Rasmus Bro from University of Copenhagen. In addition, three consecutive industrial supervisors were involved; Max Egebo, Jesper Kruse and Lars Nørgaard. In spite of the large number of supervisors, I consistently experienced an inspiring and well-functioning "supervision group", and I would like to express my thanks to all five. Søren, for enthusiastic supervision, for sharing your valuable experiences in the world of plant science and nutrition and for fruitful discussions throughout the years. Rasmus, for instant, and useful, guidance to handle chemometric complications even with the busiest calendar, and for an always optimistic view on the situation. Max, for helping convince FOSS to support the project, for being an excellent Matlab-mentor and for always being able to spare time. Jesper, the only supervisor with a business-background, thanks for giving insight into a completely different world and mindset and for valuable input when patent-discussions started. And finally Lars, thank you for being a most excellent boss as much as supervisor, whose constructive approach and sensible advice have been invaluable during the last months of this work.

Furthermore, I would like to express my gratitude to FOSS for giving me the chance to do this PhD work. For me, it has been a great incentive to have a business perspective on my work, and I have enjoyed switching between two very different work environments at FOSS and at university. I have had the pleasure of joyful and professional work atmospheres, and I would like to thank my colleagues at both places for creating these. I am especially grateful to Frans van den Berg and Anders Lawaetz for help in and around the spectroscopy labs and to Andreas Carstensen and Jens Frydenvang for a cheerful cooperation during P-experiments.

A special thanks to Anne Stenbæk and Ida Elken Sønderby for being both competent researchers and very good friends, reading and commenting my writings when most needed.

Finally, all my love to Christian, Selma and Ellen; for support and distraction.

Marie van Maarschalkerweerd

Beijing, March 2014

Summary

In a global perspective, the agricultural sector is right now facing its biggest challenge ever. The world population is rapidly increasing, and food production has to grow at the same, or higher, speed to be able to feed everyone. At the same time, the effect on environmental and natural resources must be limited. Over-fertilization must be stopped to secure aquatic environments and to reduce greenhouse gas emissions, caused by production and use of inorganic fertilizers. For production of P fertilizer, furthermore a non-renewable natural resource is used, which is expected to be exhausted within 50-100 years if the present pattern of consumption is continued. Optimizing fertilizer addition in plant production is therefore necessary to obtain a higher production along with a reduced environmental impact. To succeed with this, it is, however, necessary to enable plant producers to diagnose the nutritional status of crops during the growing season, and at a stage sufficiently early not to threaten harvest yields. The purpose of this project has therefore been to develop new methods for early diagnosis of nutrient deficiencies in plants.

In Paper I, a review, the most frequently used techniques for soil and plant analysis are described and the usability of each technique is discussed. Focus is put particularly on the newest methods for plant analysis based on fast spectroscopy, such as visual and near-infrared (Vis-NIR) reflectance and chlorophyll *a* fluorescence. These methods enable easy, fast and cheap determination of the nutritional status of plants concerning one or more nutrients. Thereby the addition of fertilizer can be managed precisely during the growing season. Insufficient validation or erroneous use, however, often results in doubtful outcomes or, in practical application, a poor use of the added fertilizer. This is presented along with a discussion of the perspectives in the new approach to plant analysis, which is possible using fast spectroscopy.

Paper II demonstrates that Cu deficient plants can be separated from healthy plants by measuring NIR reflectance directly on fresh barley leaves. The method is specific for Cu, and the condition can be diagnosed so early that it is reversible. Paper III describes a method to diagnose P deficiency in barley plants and quantify P concentration in deficient plants. It was found that the I-step in the OJIP transient, which is the outcome of measuring chlorophyll *a* fluorescence, gradually straightens and disappears as P deficiency is induced, and this is used in the method. A similar change was found in tomato plants, indicating that this could be a general effect on photosynthesis. There are further indications that also deficiencies of Mg, Cu, S and Fe may have so far unknown, specific effects on the OJIP transient. A patent application has been filed on the method, enclosed as Paper IV.

The obtained results can relatively simply be further developed into actual instruments, as both NIR and chlorophyll *a* fluorescence are already widely used methods. Such instruments can help plant producers by optimizing nutrient addition of Cu and P, and there are clear indications that similar methods for further nutrients can be identified, to the benefit of agriculture as well as environment.

Sammendrag

Set i et globalt perspektiv står landbrugssektoren netop nu over for sin største udfordring nogensinde. Verdens befolkning vokser hastigt, og fødevareproduktionen må vokse i samme, eller højere, takt for at kunne brødføde alle. Samtidig skal belastningen af miljø- og naturressourcer begrænses. Overgødskning må stoppes for at sikre vandmiljøet og for at nedbringe udslip af drivhusgasser forårsaget af produktion og anvendelse af uorganisk gødning. Til produktion af P gødning bruges ydermere en ikke-fornybar naturressource, der forventes udtømt i løbet af 50-100 år, hvis det nuværende forbrugsmønster fortsættes. Optimering af næringstilførslen i planteproduktionen er derfor nødvendig for at opnå en højere produktion samtidig med en reduceret miljøpåvirkning. For at lykkes med dette er det imidlertid nødvendigt at gøre planteproducenter i stand til at diagnosticere afgrøders næringstilstand i løbet af vækstsæsonen, og på så tidligt et stadie at det ikke truer høstudbyttet. Formålet med dette projekt har derfor været at udvikle nye metoder til tidlig diagnosticering af næringsmangel i planter.

I Artikel I, et review, beskrives de mest anvendte teknikker til jord- og planteanalyse og anvendeligheden diskuteres for hver enkelt teknik. Der er særligt fokus på de nyeste metoder til planteanalyse baseret på hurtig spektroskopi, såsom visuel og nær-infrarød (Vis-NIR) reflektans og klorofyl *a* fluorescens. Disse metoder giver mulighed for nem, hurtig og billig bestemmelse af planters næringsstatus i forhold til et eller flere næringsstoffer. Dermed kan næringstilførslen styres præcist i løbet af vækstsæsonen. Mangelfuld validering eller forkert anvendelse resulterer imidlertid ofte i tvivlsomme resultater eller, ved praktisk anvendelse, en ringe udnyttelse af den tilførte gødning. Dette belyses sammen med en diskussion af perspektiverne i den nye tilgang til planteanalyse, der er mulig ved brug af hurtig spektroskopi.

Artikel II demonstrerer, at Cu-manglende planter kan adskilles fra sunde planter ved at måle NIR reflektans direkte på friske bygblade. Metoden er specifik for Cu, og tilstanden kan diagnosticeres på så tidligt et stadie, at den er reversibel. Artikel III beskriver en metode til diagnosticering af P-mangel i bygplanter samt kvantificering af P-koncentration i planter med mangel. Det blev fundet, at I-plateauet i OJIP-kurven, der fremkommer ved måling af tidsopløst klorofyl *a* fluorescens, gradvist retter sig ud og forsvinder i takt med at P-mangel induceres, og dette udnyttes i metoden. En tilsvarende ændring findes i tomatplanter, hvilket indikerer at der kan være tale om en generel effekt på fotosyntesen. Der er ydermere indikationer af, at også mangel på Mg, Cu, S og Fe kan have hidtil ukendte, specifikke aftryk på OJIP-kurven. Der er indsendt patentansøgning på metoden, vedlagt som Artikel IV.

De fremkomne resultater kan relativt enkelt videreudvikles til praktisk anvendelige instrumenter, da såvel NIR som klorofyl *a* fluorescens allerede er vidt udbredte målemetoder. Sådanne instrumenter kan hjælpe planteavlere med at optimere næringstilførslen af Cu og P, og der er klare indikationer af, at lignende metoder for yderligere næringsstoffer kan identificeres, til stor gavn for såvel landbrug som miljø.

List of Publications

Paper I

van Maarschalkerweerd, M. & Husted, S. Plant Analysis in Agriculture, A Historical Review with Emphasis on the Newest, Analytical Developments. *Manuscript in preparation.*

Paper II

van Maarschalkerweerd, M., Bro, R., Egebo, M. & Husted, S. Diagnosing Latent Copper Deficiency in Intact Barley Leaves (*Hordeum vulgare*, L.) Using Near Infrared Spectroscopy. Journal of Agricultural and Food Chemistry, 61: 10901-10910, 2013.

Paper III

van Maarschalkerweerd, M.*, Frydenvang, J.*, Carstensen, A., Husted S. Using Chlorophyll *a* Fluorescence to Determine Latent Phosphorus Deficiency in Plants. *Manuscript in preparation.*

**Contributed equally to the work*

Paper IV

Method and Device for Determining a Nutritional State of a Plant. *Patent application* (2013).

List of Abbreviations

AAS	Atomic Absorption Spectroscopy
AES	Atomic Emission Spectroscopy (also known as OES)
APase	Arabidopsis Purple acid phosphatase
CCD	Charge Coupled Device
CEC	Cation Exchange Capacity
CS	Continuum Source
DGT	Diffusive Gradients in Thin Films
DRIS	Diagnosis and Recommendation Integrated System
DW	Dry Weight
F-AAS	Flame ionization AAS
Fd	Ferredoxin
FIA	Flow Injection Analysis
GF	Graphite Furnace
ICP	Inductively Coupled Plasma
LIBS	Laser Induced Breakdown Spectroscopy
LV	Latent Variable
MIR	Mid-Infrared
MS	Mass Spectrometry
MSC	Multiplicative Signal Correction
NA	Nicotianamine
NBI	Nitrogen Balance Index
NDVI	Normalized Difference Vegetation Index
NIR	Near Infrared
NUE	Nutrient Use Efficiency
OES	Optical Emission Spectroscopy (also known as AES)
PC	Principal Component

PCA	Principal Component Analysis
PLS	Partial Least Squares
PLS-DA	Partial Least Squares – Discriminant Analysis
POCI	Potato Oligo Chip Initiative
PQ	Plastoquinone
RF	Radio Frequency
RMSECV	Root Mean Square Error of Cross Validation
RMSEP	Root Mean Square Error of Predictions
RPD	Ratio of Prediction to Deviation *or* Residual Prediction Deviations
SFA	Segmented Flow Analysis
SPAD	Soil-Plant Analysis Development
UV	Ultra Violet
Vis	Visual
XRF	X-Ray Fluorescence
YFDL	Youngest Fully Developed Leaf

Contents

Chapter 1

Introduction

Background

During the last 10,000 years, the environment of our planet has been in an unusually stable period, where environmental conditions, including temperatures, have stayed within a narrow range. This stability has enabled the development of agriculture and of human civilization as such. However, a thorough investigation of the status of our planet states that the climate change, changes in N and P cycles and biodiversity loss are already so serious that we are endangering the stability of our environment. This is due to our heavy reliance on fossil fuels along with the industrialization of agriculture (Rockström et al., 2009). Production of N fertilizer is the single most energy consuming process in agriculture, using 1.4-1.8 liters diesel fuel per kg N (McLaughlin et al., 2000), and it is thereby a major contributor to the increased atmospheric concentration of greenhouse gases. Production of P fertilizer is less energy demanding, however, raw phosphate rock, the raw material for P fertilizer, is a non-renewable resource that is rapidly dwindling. In as little as 50-100 years, no clean reserves of phosphate rock may be left, and agricultural production cannot continue in its present form (Gilbert, 2009). Over-fertilization of both N and P, provided as organic or inorganic fertilizer, has devastating effects on land and water resources, affecting eco-systems as well as human access to clean water (Gao et al., 2006;Chen et al., 2008;Wang et al., 2011). Finally, the inclusion of more and more land for agriculture has a negative impact on biodiversity and thereby on fragile ecosystems (Rockström et al., 2009). It is therefore evident that present agricultural practices must be changed to bring fertilizer and energy consumption significantly down.

At the same time, the world population is increasing rapidly. Estimates of rises in food demand go as high as 100% from 2005 to 2050 (Parry and Hawkesford, 2010;Tilman et al., 2011), and to meet this demand, enormous agricultural yield increases must be obtained. What we demand from agriculture is, thus, a spectacular increase in efficiency. Outputs must be increased extensively, using significantly less input. This is a challenge that can only be met by making major efforts in both scientific and political fields, but one part of major importance is providing the tools that enable plant producers to optimize nutrient management.

Reaching the optimal point of fertilization, where every grain of fertilizer pays off in increased yields takes attentive monitoring of plant development throughout the growing season to provide crops with the right fertilizer at the right time and in the right amount. Timing is important in terms of fertilizing before a given deficiency causes permanent damage, and for environmental reasons to ensure timely uptake of especially N to avoid leaching and

eutrophication. Soil analysis is commonly used to predict crop fertilizer needs, but these methods are not applicable for monitoring during the growing season and cannot be used to test for acute nutrient deficiencies. Furthermore, there is often a poor relation between analytical results and actual plant available nutrients (Mason et al., 2010). Traditional plant analysis in a laboratory is costly, and sampling, handling and analysis time before a result is given are all time consuming steps. Furthermore, interpreting the results and deciding on the required action is not always straightforward.

It is evident from the above that a different approach is needed. There is a need for methods that deliver instant assessments of plant nutritional status and which can determine plant needs with all the variation that occurs, throughout fields and growing seasons. Such methods are not a dream scenario found in a far-away future. They can be obtained using fast spectroscopy in agriculture, and to some extent, this is already done. Several tractor-mounted instruments for on-the-go assessment of N nutritional status in crops using Visual-Near Infrared Reflectance (Vis-NIR) spectroscopy are used in practical agriculture (e.g. NTech, 2013;Yara, 2013), and corresponding hand-held instruments determine similar indices of N status. Also Mn status can be determined spectroscopically, using chlorophyll *a* fluorescence (NutriNostica, 2013), as well as chlorophyll and anthocyanin concentrations, to decide the optimal harvest time of grapes, using Ultra-Violet (UV) - Vis reflectance and fluorescence (Force-A, 2013), to mention a few. These instruments deliver instant results, which are translated into plant status. For the tractor-mounted N-sensors, a direct link to fertilizer applying equipment on the same tractor enables immediate action.

Fast, spectroscopic methods have a number of advantages in addition to the speed of analysis. They are generally cheap once the equipment has been acquired, environmentally friendly, as no toxic reagents are needed, easy to use and able to deliver consistent results if used and interpreted appropriately (Samborski et al., 2009;Schmidt et al., 2013). These are all reasons for their extensive use in other fields such as pharmaceutical and food industries. Though solutions for analysis of plant nutrition exist, the range of targeted nutrients is still very limited. Furthermore, specificity of the methods is a major issue, which may lead to misinterpretations of results, as it has been demonstrated for N-sensors (Zillmann et al., 2006;Samborski et al., 2009).

The idea behind this PhD study was to identify and describe new methods for diagnosing latent nutrient deficiencies in plants using fast, spectroscopic techniques. Measuring directly on fresh leaf samples was prioritized to minimize time consumption in the final methods. Combinations of a number of relevant plant nutrient deficiencies and an array of spectroscopic techniques were screened to investigate possible correlations.

Objectives

The main objective of this thesis was to investigate and develop new methods to diagnose nutrient deficiencies in plants using fast spectroscopic techniques applied directly on fresh leaves. Focus was put on essential plant nutrients, micro- or macro-nutrients, of which deficiencies are common, on a global or local scale. The tested spectroscopic methods included near infrared reflectance (NIR) and time-resolved chlorophyll *a* fluorescence. Barley (*Hordeum vulgare*, L.) was chosen as a model plant.

Each diagnostic method must eventually fulfill the following criteria:

- *Deficiency can be diagnosed already at a latent stage, meaning before any visual deficiency symptoms occur.*
- *Deficiency must be reversible at the earliest time of diagnosis, thereby indicating that no permanent damage to plant metabolism has occurred.*
- *The method must be specific for a given nutrient,* i.e. *deficiency of another nutrient may not disturb the results.*

Elements are not spectroscopically active in themselves. Given the complexity of plant stress reactions, and the number of metabolites that are affected, an empirical approach was therefore chosen to reveal specific correlations between spectroscopically active components reflecting the nutritional status of plants.

Thesis Outline

This thesis consists of an introductory part (**Chapters 1-5**), of which **Chapter 2** is a review paper (**Paper I**), followed by another two scientific papers (**Papers II and III**) and a patent application (**Paper IV**).

Paper I is a review concerning plant analysis. It contains an overview of the history of plant analysis and the major challenges in the field of plant nutrition today. The differences in the approaches of plant and soil analysis are evaluated. Emphasis in the review is on analytical methods for assessment of plant nutritional status, which are described with a main focus on fast, spectroscopic methods, of which future perspectives are also discussed.

Near Infrared spectroscopy, chlorophyll *a* fluorescence and the applied methods for data analysis are described (**Chapter 3**), and the contributions to data variability are investigated and discussed (**Chapter 4**). Finally, conclusions and perspectives of this PhD study are summarized (**Chapter 5**).

Paper II describes the finding of a specific correlation between NIR spectra and Cu deficiency in barley.

Paper III describes the finding of a specific change in chlorophyll *a* fluorescence during P deficiency in barley. The method is indicated to relate to plants in general.

Paper IV is a patent application based on the findings described in **Paper III**.

Chapter 2

Plant Analysis in Agriculture, A Historical Review with Emphasis on the Newest, Analytical Developments (Paper I)

van Maarschalkerweerd, M. & Husted, S.

Manuscript in preparation

Plant Analysis in Agriculture

A Historical Review, with Emphasis on the Newest, Analytical Developments

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Abstract

Yield optimization and thereby plant nutrition has been a main focus for plant producers since the beginning of farming, and long before the concept was even described. Today, a rapidly growing world population and thereby a vastly increasing food demand requires significant agricultural yield increases worldwide. At the same time, sub-optimal or excess use of fertilizers lead to severe environmental damage in areas of intensive agricultural production. Optimal management of fertilizer input is therefore more relevant than ever, and for this, plant analysis plays an essential role.

Here, the approaches of soil and plant analysis are compared and discussed, with emphasis on analytical techniques for plant analysis. State of the art methods for total analysis of elemental concentrations in plants, Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) and – Mass Spectrometry (–MS) and Atomic Absorption Spectroscopy (AAS), are described along with brief accounts on historical methods. The main focus of this review is on new techniques using fast spectroscopy that offer cheap, rapid and easy-to-use analysis of plant nutritional status. The majority of existing methods uses vibrational spectroscopy, such as Visual-Near Infrared (Vis-NIR) and to a lesser extent Ultraviolet (UV) and Mid-Infrared (MIR) spectroscopy. Advantages of and problems with application of these techniques are thoroughly discussed. Spectroscopic techniques considered having large potential, such as X-ray fluorescence (XRF) and Laser-Induced Breakdown Spectroscopy (LIBS) are also briefly described.

Contents

1 Introduction

Plants are photoautotrophic organisms, meaning that they acquire energy from sunlight for carbon fixation. By using water, CO_2 and 14 essential mineral nutrients, they are able to synthesize all chemical components needed to complete a full life cycle, from the germination of a seed to the production of new, mature seeds. The essential mineral nutrients are divided into two groups, macro- and micronutrients, according to the amount generally required by plants. The six macronutrients, N, P, K, S, Ca and Mg, are present in g/kg of plant dry matter. The eight micronutrients, Fe, Mn, B, Zn, Cu, Ni, Mo and Cl, only in mg/kg dry matter. In spite of the great concentration differences, all 14 nutrients are irreplaceable in all plant species, as each fulfills one or more specific tasks within plant metabolism (Kirkby, 2012). In addition to the essential mineral nutrients, a number of elements are designated as beneficial, of which the most thoroughly investigated are Al, Co, Na, Se and Si. The beneficial elements are not required for all plants but may promote growth in specific taxa, in some cases even be essential for certain species (Pilon-Smits et al., 2009).

1.1 Plant Nutrition in Ancient Times

Nutrient management has been a main concern in plant production all over the world since the beginning of farming. The concept of plant nutrition was unknown for millennia, but still management practices were developed in ways that optimized the nutrient availability for crops. One of the earliest cropping systems known is slash-and-burn, a system of shifting cultivation. Woodland is cleared and smaller bushes are burnt, leaving a nutrient rich layer of ashes in the top soil. The land now gives higher yields for a few years, after which new land has to be cleared (Fussell, 1971). The system was developed independently in several parts of the world and was for instance used by Native Americans (Anderson, 2005) as well as in Europe already in the stone age, *i.e.* before year 2000 BC. It is efficient and as such sustainable, if only the land is left fallow for sufficiently long time after cultivation. Thus, when population density increases above a certain threshold, slash-and-burn will lead to exhaustion of soils, as it is seen *e.g.* in the Brazilian Amazon and North East India, where the technique is still used today (Comte et al., 2012;Goswami et al., 2012).

Probably the first mention of manuring of soil occurs in "Oeconomicus", written by Xenophon in classical Greece around 362 BC. The practice of burning stubbles is described together with the positive effects of weeding and turning in young weeds (Fussell, 1971). According to the publication, an evaluation of the natural vegetation on a given soil could give the farmer an idea of which crops would be best fitted for growing on that location, and the Roman poet Virgil (70-19 BC) even developed a number of simple soil tests leading to recommendations of crop selection. Guidance concerning agricultural practices occurs in many publications from ancient Greece and Rome, but common for all advice and theories from these times is that no actual reasons for the benefits are explained as they were at the time unknown. The unusual fact that a poet gave advice on agriculture emphasizes that actual scientific approaches in agriculture had not yet been introduced.

The Roman writer, farmer and politician Cato (234-149 BC), was the first to write a book specifically on farming in Latin, "De Agricultura". He explained the making and using of a dung heap and thoroughly described the use and positive influence of legumes (Dalby, 1998). It should be kept in mind that though theories and knowledge were written down and instructions were given in print, the actual users, the farmers, were illiterate. Furthermore, there was the matter of distribution; as printing had not yet been invented, the number of books was very limited. Therefore, ancient writings on farming should be regarded merely as descriptions of best management practices used at the time. For two thousand years, from the Ancient Greeks to the invention of printing, plant science was more or less static. Not only because of the lack of basic knowledge on which plant science could be developed, but also because medicine was favored in science. At one point, the knowledge of Greek language diminished, almost disappeared, due to the Roman dominance and thereby the introduction of Latin. In spite of Catos work, much experience, assumptions and ideas concerning plant cultivation was hereby lost (Fussell, 1971).

1.2 Emergence of Agricultural Science

Until the beginning of the nineteenth century, real quantum leaps in the science of plant nutrition are not found. Then came the German agronomist Albrecht Thaer (1752-1820), who is regarded the founder of agricultural science. He introduced the use of experimental methods as pot and field experiments and thereby facilitated a change from agricultural knowledge based

on a collection of empirical experiences to an actual science, testing hypotheses experimentally in a systematic fashion. He was also one of the main persons developing and describing the so-called “humus theory”, which was widely accepted at the time. The main assumptions of this theory were that the majority of plant dry matter, known to be C, was derived from so-called “nutritive juices” in the humus layer of the soil. Secondly, it was recognized that the nutrient demand of a plant depended on plant species (Feller et al., 2003;Manlay et al., 2007). With some exceptions, using the extended principles of the humus theory resulted in agricultural advice of high quality. The major problem was that the concept of mineral nutrition had not yet been discovered, and the humus theory was based on the false assumption that C was derived from the soil. This was soon to be corrected by a student of Thaer’s, Carl Sprengel (van der Ploeg and Kirkham, 1999;Feller et al., 2003).

Carl Sprengel (1787-1859) continued Thaer’s investigations of humus extracts and the humus theory. However, he concluded that the enhanced plant productivity, which is observed when increasing the humus content of the soil, is not a result of the humus itself but of the mineral nutrients contained in it. Thereby, in 1828 he introduced the theory of mineral nutrition of plants. As a consequence of this, he also suggested the Law of the Minimum: *“When a plant requires twelve* [all essential plant nutrients had not been discovered, and some were erroneously considered essential] *substances for its development, it will never grow if only one of this number is missing, and continually grow miserably when it is not present in a quantity the plant requires”*. The mineral theory and the Law of the Minimum are usually attributed to Justus von Liebig (1803-1873), another German chemist, who presented the two in 1840 and 1855, respectively, as his own discoveries, despite being well acquainted with the work of Sprengel. Liebig was an outstanding communicator and a pioneer in agricultural science, and he spent a considerable part of his career fighting for the acceptance of the ideas of mineral nutrition. The agricultural community, including research stations, was not eager to accept the entrance of chemistry into the agricultural field, but as history shows, they were eventually convinced. This intensive effort is probably the reason why Liebig, and not Sprengel, is the person remembered for the discoveries. (Finlay, 1991;van der Ploeg and Kirkham, 1999;Jungk, 2009).

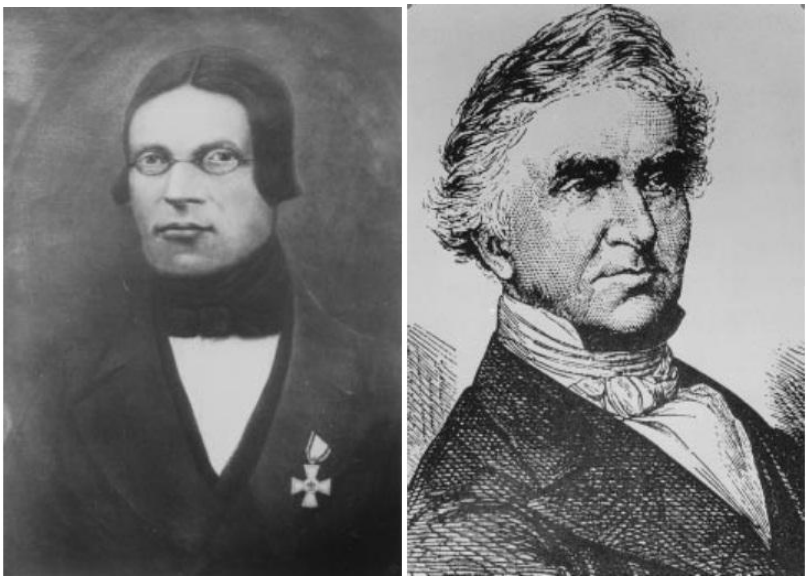

Figure 1. Carl Sprengel (1787-1859) (left) and Justus von Liebig (1803-1873) (right). From van der Ploeg and Kirkham (1999).

1.3 Productivity Leaps in the 20th Century

Around year 1900, nearly no fertilizer input was provided for agricultural fields, causing a notorious shortage of N. Nitrogen input came almost exclusively from N-fixing crops, crop residues and human or animal wastes, and a diminutive amount of guano, N rich bird droppings imported from Chile, used as a fertilizer. Then, in 1909, Fritz Haber invented a method for synthesizing ammonia from atmospheric nitrogen. This was rapidly developed into an industrial production of synthetic N fertilizer by the engineer Carl Bosch, why the process was named the Haber-Bosch process. Already in 1913, large scale production was possible, and the use of synthetic fertilizer in agriculture started. However, it did not accelerate until the 1950's. World War I and economic setbacks in the 1930's first limited the application. Then, during World War II, much ammonia was used as explosives instead of as fertilizer, and a large number of production plants were destroyed, primarily in Europe and Japan. Hence, though US production increased solidly, the global production of ammonia in 1945 was lower than by 1920. However, steep increases in production and consumption of ammonia after the war lead the way for harvest yield increases, and it ended food rationing in Europe (Smil, 1991;Smil, 2004).

Rough calculations on fertilizer input and yields in year 1900 compared to present show that around 40% of today's world population is alive, thanks to the Haber-Bosch process and the increased yields it facilitates. In the western world, the use of N today could easily be limited by reducing the excessive protein consumption from meat-based diets in favor of a higher consumption of plant products. But in low-income, densely populated countries, many found in Asia, N fertilizer is often what keeps the populations away from hunger (Smil, 2002). It is, thus, evident that synthetic N fertilizers are indispensable for maintaining the lives of the world population today, and the Haber-Bosch process was one of the main factors enabling the success of the Green Revolution.

1.3.1 The Green Revolution

In spite of the higher yields obtained through the increased use of N fertilizers, more than one third of the world population suffered from malnutrition in the late 1950's. In Asian, African and Latin American countries, famine threatened millions. This caused farmers, scientists and policy makers to join forces to create a tremendous yield increase in major food crops such as rice, maize and wheat obtained over roughly 25 years; this was coined "the Green Revolution". New varieties were bred, featuring much higher yields due to a number of factors including short growth duration, resistance to biotic and abiotic stresses, and superior grain quality. The most important breeding achievement is considered to be the introduction of the so-called dwarfing genes. Supplying high rates of N fertilizer to cereal varieties from before the Green Revolution resulted in excessive vegetative growth, and the elongated stems were not strong enough to support the weight of the panicles. This caused lodging and ultimately lower yields than when poorer nutrition was provided. Introducing the dwarfing genes resulted in shorter and stiffer cereal straws as well as increased tillering. By optimizing nutrition to these varieties, nutrients were directed mainly to grain production, and the increased panicle weight could be carried by the straws without lodging. The introduction of dwarfing genes alone was able to increase the harvest index, *i.e.* the ratio of grain dry weight to total dry matter, by 60% in rice and wheat varieties (Khush, 2001). Combining these new varieties with improvements in fertilization and other management practices such as irrigation released an enormous yield potential, leading to a doubling of agricultural yields in Asia from 1965-1990 (Khush, 2001;Hazell, 2009).

Unfortunately, public investments in agricultural research and development decreased significantly, globally seen, after the Green Revolution, causing yield growth to slow down towards the end of the millennium. As an example, the average global yield growth in wheat was 2.1% annually during the 1970's, whereas in the 1990's the number was reduced to only 0.4% per year (Gruhn et al., 2000). Importantly, and unfortunately, the decline in yield growth is most pronounced in the poorest countries (Hafner, 2003).

1.4 Major Challenges of Today

In spite of the achievements of the Green Revolution, tremendous challenges are still faced by the agricultural community today - and by the world. The world population increases rapidly and is expected to continue to do so for another forty years, putting further pressure on food production. By 2050 the world population is forecasted to reach 9 billion people, and estimates of increases in food demand from 2005 to 2050 are as high as 100% (Parry and Hawkesford, 2010;Tilman et al., 2011). At the same time, climate changes alter local temperature and precipitation conditions. In most locations this will challenge yields and management practices, and farmers will have to learn how to adapt to these changes, hopefully assisted by renewed plant breeding efforts (Nelson, 2010). Nutrient management is a major concern for future agriculture. At present, over-fertilization causes severe environmental damage, mainly in North America, China and Europe, while in other parts of the world, nutrient deficiencies limit yields severely (Gruhn et al., 2000;Chen et al., 2008). Especially in the poorest regions of the world, including sub-Saharan Africa, critical nutrient depletion of soils is common. Wind and water erosion remove the most fertile layers of the soil in many areas, and the amount of nutrient input to the land is generally far from what is removed, causing soil degradation and even desertification. In combination with increasing populations, this has made it necessary many places to include marginal and fragile soils in the farmed area (Gruhn et al., 2000). In spite of these inclusions, the worldwide area of cultivated land per person gradually decreased in the period 1961 – 2009 from a world average of 0.37 to 0.20ha, a 44% decrease, substantially larger in the poorest countries, which further stresses the need for increasing productivity (WorldBank, 2012).

1.4.1 Fertilizer Challenges

The increased use of inorganic fertilizers comes at a price, as the production of especially N fertilizer is highly energy consuming. For one kg of N, 1.4-1.8 liters of oil is used, or about ten times the energy used for P and K fertilizer production (Kongshaug, 1998;McLaughlin et al., 2000). Investigating fertilizer prices over the last 30 years therefore shows a close correlation with the price of crude oil and accordingly an expectation of continued price increases in the future (Figure 2).

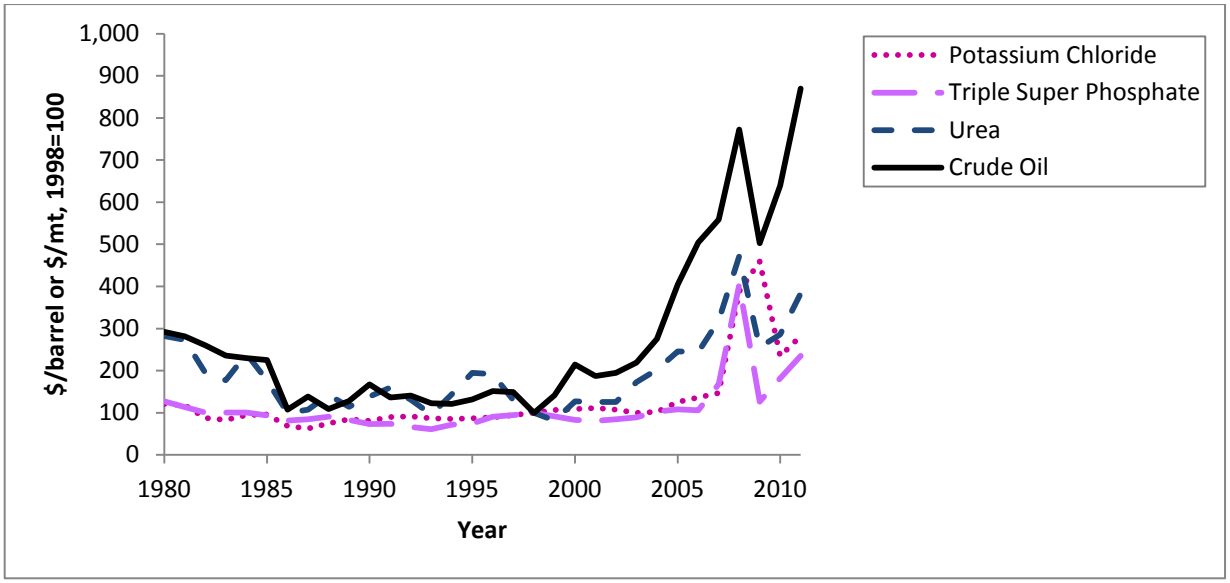

Figure 2 Fertilizer prices ($/metric tonne, normalized to 2005$) and crude oil price ($/barrel) from 1980-2011, with 1998=100. Compiled from WorldBank (2012) and BP (2012).

Along with the financial price of fertilizer consumption come the environmental costs, including the negative effects on climate due to vast greenhouse gas emissions. Quantifications of greenhouse gas emissions are available but vary widely depending on how much of the total life cycle is included – transportation and distribution of fertilizer may or may not be included, as well as the number of gases investigated may differ. It is, however, agreed upon that by substituting older production plants for new technologies, both energy consumption and greenhouse gas emissions can be significantly reduced. European averages of CO_2 equivalents emitted per kg N produced are around 7kg. For modern technologies alone, the average is around 3kg, a substantial improvement (Kongshaug, 1998;Wood and Cowie, 2004).

In addition to the vast energy consumption and greenhouse gas emissions, productions of P and K fertilizers exploit finite natural resources. For P this is a major problem in near future with the most pessimistic forecasts saying that global resources of clean phosphate rock will be gone within 50 years (Gilbert, 2009). The largest part of the world's total remaining phosphate rock, 75%, is found in Morocco and Western Sahara. China possesses around 6%, and South Africa, Russia, USA, Algeria, Jordan and Syria each have 2-3% (USGS, 2013). This geographic concentration of the scarce resource may further increase a global shortage, causing even higher prices or being used in political disagreements. One solution to this problem is to find alternative sources of fertilizer. An example of this is the use of $MgNH_4PO_4 \cdot 6H_2O$, or struvite, a phosphate mineral that can be precipitated from waste water and preprocessed into fertilizer (Linderholm et al., 2012). Another promising approach is to focus on nutrient use efficiency (NUE) in plant breeding, improving the NUE of specific genotypes. However, also intercropping has proven very efficient. Some plant species acidify the rhizosphere and thereby increase the solubility of P, and especially legumes produce root exudates which enhance P solubility by chelating metals that bind P in soil. It has been demonstrated that species with low P efficiency, *e.g.* cereals, may benefit greatly from intercropping with legumes (Li et al., 2007).

1.4.2 Challenges for Breeding

A plant with improved NUE is defined as one producing more yield per unit nutrient, applied or absorbed, than other plants under similar growth conditions (Fageria et al., 2008). This includes plants that are better at absorbing nutrients from the soil as well as those better at utilizing what they have absorbed. Thus, improving the NUE of plants with respect to one or more essential plant nutrients will increase yields without increasing input level, and especially concerning the problem of dwindling P resources this will be a sustainable solution.

Intensive work is carried out in the hunt for new crop varieties. During the Green Revolution, focus was mainly on enabling plants to make use of improved management practices, especially increased inputs. Today, the aims for new varieties may vary more broadly, as improved cultivars are needed both for sustainable crop production on marginal lands as well as for increasing yields even more on the most fertile soils. Molecular plant breeding is being used as a tool to control specific processes in the plant by targeted genetic manipulation (Parry and Hawkesford, 2010). In soybean, the NUE for P has been improved by expression of *AtPAP15*, an Arabidopsis (*Arabidopsis thaliana*) purple acid phosphatase (APase) gene, which increases the activity of APase and phytase in roots, thereby enhancing the mobilization of inorganic phosphate from organic P sources in the soil. The expression of the gene caused plant P concentrations to increase with between 18 and 90%, and dry weights increased by between 57 and 118% as compared to control plants (Wang et al., 2009). Investigations of the genome of traditional rice varieties originating from regions with soils poor in P have recently lead to the identification of a protein kinase gene named *PSTOL1*, for phosphorus starvation tolerance. Overexpression of *PSTOL1* increases early root growth and thereby enhances the plant's ability to mine soil P reserves. Grain yield increases of more than 60% were found in modern, P-starvation intolerant varieties, where the gene was overexpressed, when cultivated in P deficient soils (Gamuyao et al., 2012). These new findings could potentially lead to significantly higher yields for plants cultivated under P limiting conditions.

More than two billion people suffer from Fe deficiency worldwide, leading to massive health problems as well as decreased learning abilities for children and reduced work productivity levels. Iron levels in the grains of cereals such as rice are generally very low, even in well-supplied plants. However, it has been demonstrated that by upregulating the synthesis of nicotianamine (NA) in rice plants, the Fe level in the plant was increased. Specifically in the grain, the concentration of bioavailable Fe was shown to be at a significantly higher level than in control plants. This finding could potentially benefit millions of people globally, who eat rice as their stable, and main, food (Lee et al., 2009).

In spite of large numbers of encouraging results, commercializing transgenic cultivars is still extremely difficult. Genetic engineering is not always robust and easily reproducible, legal issues can be complicated and in addition to this, the public opinion is generally opposed to these varieties.

1.4.3 Optimization of Nutrient Management

Whether focusing on development of improved crop cultivars or new sources for fertilizers, optimization of nutrient management is more than ever a pivotal point in farming. Maintenance, and improvement, of soil fertility is crucial to obtain the highest yields that are needed to feed the growing world population, and whilst marginal soils are increasingly included as agricultural

lands, the world cannot afford deterioration of fertile lands with poor management. To optimize fertilization practices, it is therefore essential for plant producers to know the fertility characteristics of their soil as well as to monitor the development of plants throughout the growing season. This way it is possible to act in accordance to plant needs instead of acting in accordance to tradition or interpretations of analyses, which may be invalid. In order to pursue this purpose, cheaper and more easily accessible methods for plant and soil analyses are crucial. Today, soil analysis is by far the most commonly used, as a "predictor" of crop fertilizer needs in the coming growing season. Plant analysis is more difficult to use, as sample handling is more demanding, and analyzing and using the results correctly in fertilizer management is still challenging.

This paper compares soil and plant analysis and reviews the development of methods for plant elemental analysis from the first, gravimetric methods and up to today's state of the art methods including emerging, spectroscopic methods to be used directly in the field.

2 Plant and Soil Elemental Analysis

Producing a high yield of the best possible quality has always been a goal for plant producers. In modern agricultural practice, nutrient management practices as fertilization and crop rotations are essential tools to obtain this. Crops need to be provided the right nutrients in adequate amounts while at the same time avoiding over-fertilization, both for economic and environmental reasons. Plant and soil analyses are indispensable tools to assist the plant producer in this.

Presently, and historically, soil chemical testing has been widely used. For assessing fertility of soils as well as soil contamination, no technique has been applied as much as soil testing (Rayment, 1993). However, it is not without dispute, since the methodologies carry serious problems regarding both practical performance of the tests as well as interpretation and use of the results. Knowledge about physical characteristics of the soil can assist the plant producer in fertilization management, as these affect the nutrient availability for crops. But they are far from the only factors of importance, why additional analysis is essential for optimal yields. Traditionally, soil chemical testing has been the method of choice, but plant analysis is increasingly gaining ground as these methods are rapidly improving in speed, precision and cost, and the weaknesses of traditional soil testing become more widely acknowledged.

The main difference between soil and plant analysis is plainly the scope of the methods. Where soil analysis is aimed at obtaining predictions for fertilizer needs in coming growing seasons, plant analysis provides information about the current nutritional status and enables diagnosis of acute disorders during the season. Both approaches are reviewed in this chapter, with focus on advantages and disadvantages, limitations and possibilities of each technique. In many cases, it is not necessarily a question of choosing one approach or the other. If using state of the art methods, combining soil and plant analysis may be the best way to obtain optimal nutrient management of a crop.

2.1 Soil Elemental Analysis

Agricultural soils are analyzed with the purpose of assessing the potential nutrient release of the soil for crops in the coming growing seasons and thereby allowing the plant producer to estimate fertilizer needs in advance. Both physical and chemical characteristics are analyzed for, as they are, to some extent, correlated. Soil contents of sand, silt, clay and organic matter are determined as well as Cation Exchance Capacity (CEC), pH and nutrient concentrations. Combining these results, fertilizer recommendations are developed. Soil testing has been widely accepted and used for this purpose since the 1940's (Westerman, 1990), and today, agricultural fields are routinely analyzed, commonly every 3 to 5 years. Thus, modern plant producers often have a long historical record of their fields (Pedersen, 2012). Recommendations are given based on the assumption that the determined nutrient concentrations correlate with, or represent, the plant available portion from which the needed fertilizer addition for a given crop can be calculated. However, as will be discussed, this is rarely the case.

A number of advantages have lead to the wide use of soil analysis in agriculture. Sampling is, conveniently, carried out during the less busy seasons when there are no crops in the field, *i.e.* after harvest or before sowing. The possibility of planning fertilization ahead is practical for the plant producer, and finally there has been a lack of accessible alternatives. The obvious alternative is plant analysis, which will be discussed in detail later, but it has for long not been able to compete with soil analysis in terms of price, ease of use and perceived benefits, why soil analysis has remained the dominating tool for fertilizer management in practical agriculture. To demonstrate the proportions, 1,011 plant analyses were collected and analyzed in agricultural fields in Denmark in the growing season 2011. During the same period, 75,252 soil samples were analyzed (Pedersen, 2011). However, major disadvantages of soil analysis are the wide array of methods employed and the time consumption per sample, leading to excessive workloads in laboratories as well as difficulties in comparing values across countries or regions (Rayment, 1993). Even more importantly, larger investigations of soil analytical methods have revealed a poor or complete lack of correlation between results and early stage dry matter yields or plant nutrient concentrations. This will be elaborated below.

A soil analysis for a given nutrient is basically carried out by agitating the dried, ground soil sample with an extractant for a certain period of time, filtering and finally analyzing the filtrate for concentration of the nutrient or derived compounds. Key considerations during soil analysis are not only the extractant but also sampling, soil preparation (typically drying and grinding), extraction method, analysis, quality assurance and control (Rayment, 1993). Ideally, the results of a soil analysis should be independent from who carried out the sampling, and which laboratory performed the analysis. Ensuring this needs careful standardization of all procedures, as artifacts may arise from all types of handling and storage.

2.1.1 Sampling

Due to the heterogeneity of fields, sampling is extremely important for soil analysis. If carried out in an inappropriate way, the final results of the analysis can be useless. Soil samples are collected during the period after harvest or before a new crop is established. No fertilization, liming or similar can be carried out before sampling. Fertilizer requirements may vary enormously over a field or even within small areas of the field, however, for practical reasons every square meter cannot be measured. Unless a field is known not to vary in texture, yield or

cultivation history, GPS marked sampling is a useful tool to ensure meaningful sampling. In Denmark, between 50 and 75% of the total number of soil samples collected are GPS marked, enabling the plant producer to vary application rates over a field. This practice assists in obtaining more uniform yields and better NUE (Pedersen, 2011; 2012). Based on maps showing yield variations, cultivation history (*i.e.* historic boundaries of fields), topography and results from previous soil analyses, fields are divided into subfields of relative uniformity. In each subfield, at least one sample is taken, consisting of 16 subsamples collected following a specific pattern, typically a diagonal across the subfield, as shown in Figure 3 (Anonymous, 2003).

Figure 3. One field divided into nine subfields. From each subfield, 16 soil samples are collected (marked by stars) according to the pattern marked by the red lines. The subsamples from each subfield are combined into one sample for analysis. Figure from Anonymous (2003).

An alternative method to the subfields is to make a quadratic grid in the field, with sides of 75m (2 samples per ha) or 100m (1 sample per ha) and sample diagonally across the squares. The GPS positions of samples are marked at the middle of the sampling routes, enabling the plant producer to adjust management practices according to these results (Hansen, 2002).

Sampling depth depends on the desired nutrient analyses as well as tillage systems and crops. Sampling exclusively the plough layer, 0 - 15cm, may be adequate for analysis of less soil mobile nutrients as *e.g.* P or K. If no-tillage systems have been applied for long, deeper sampling down to *e.g.* 30cm is advisable even for such nutrients, as they may become stratified. Water-soluble, highly soil mobile nutrients such as NO_3-N and S on the other hand require sampling down to 60cm. To determine the stratification of such nutrients, sampling is commonly split into two, from 0-15cm and from 15-60cm. The quality of some crops, as *e.g.* sugar beet, is very sensitive to the level of NO_3-N, why sampling below 60cm in some cases may be required to adjust fertilization precisely (Franzen and Cihacek, 1998;Pennock et al., 2008).

For some specific cultivations, soil analysis is impractical. The root zone of deep-rooting species as fruit and forest trees is difficult to assess, and in addition often very deep. This makes it practically impossible to collect proper samples to give a picture of the nutrients available to trees (Römheld, 2012). In such cultivations, plant analysis is more commonly used to obtain information about fertilizer needs.

2.1.2 Sample Handling

Drying of samples must be carried out in clean drying equipment at uniform temperatures, and subsequent grinding should be to a standardized size, which will commonly mean passing through a 2mm sieve (Rayment, 1993;Plantedirektoratet, 1994). However, particle sizes may differ between methods, as the optimal sizes vary. Particle size has been shown to affect analytical results significantly, why variation should be avoided if comparable results are wanted (Rayment, 1993).

Storage time and temperature are of little or no significance in most cases, but measured S concentrations have been shown to change over time, as well as measured P concentrations may increase with as much as 50% in the same soil when stored at 36°C as compared to storage at 4°C (Rayment, 1993). Hence, standardizations are required also for storage conditions, if comparable results are to be obtained in different laboratories.

2.1.3 Extraction and Analysis

Innumerous extractants in many different concentrations and at various pH levels are used in soil analysis, with each laboratory typically providing one or a selected few for each nutrient analysis. Extractants can be divided into categories, each dissolving different fractions of nutrients in the soil. Water alone extracts only already dissolved ions, whereas dilute salt solutions as KCl and $CaCl_2$, very dilute acids and aqueous organic solvents extract soluble salts as well as adsorbed ions and salts, which are readily available for plants. Chelates, *i.e.* buffered EDTA and DTPA solutions, can be used for extracting adsorbed and complexed nutrients. Finally there are the more aggressive extractants as strong acids, including *aqua regia*, a mixture of HNO_3 and HCl used for determination of "total concentrations", *i.e.* the sum of the natural background concentration of the soil and fertilizer applications in the past. Using different extractants, results for the same soil sample can vary as much as from 4.2mg P/kg ($CaCl_2$) up to 478.4 mg P/kg (*aqua regia*), the latter of which is of very little practical use, as most of it will be inaccessible for plants (Rayment, 1993;Gassner et al., 2002).

The results of many extraction methods co-vary to a high degree, as the differences, chemically seen, can be very small. The same extractant may be used in different concentrations, or soil to extractant ratios, extraction times and extraction temperatures may vary. Extractants belonging to the same category will commonly yield highly related results (Houba et al., 1996;Mason et al., 2010). There is no uniformity, globally nor locally, concerning extractants and extraction methods used in soil analysis. In 1863 a commission of agricultural chemists was appointed in Germany to settle on uniform methods for soil analyses. They did not succeed, mildly put, as now, 150 years later, the analytical processes employed still vary widely between countries and even regions. As an example, to test for plant available P in soil, Fixen and Grove (1990) list 12 different extractants, which are all commonly used in soil test laboratories. There are a number of reasons for this. During 1930 – 1970, a large number of analytical methods were developed by individual research workers, with little or no co-ordination (Houba et al., 1996). Chemical and

physical characteristics and problems of soils may have wide spatial variation, and test methods were generally optimized for local soil conditions of the developing research worker. Laboratories tend to prefer quick and cheap tests in order to be able to compete on prices, and finally there is an ongoing quest to improve analytical methods, which is in many ways very positive. It does, however, all in all result in a huge variation in methods employed, which makes meaningful exchange of information and experiences across national or regional borders very difficult (Rayment and Lyons, 2012).

Using one single extractant for analysis of all or most relevant nutrients would save much time in the individual laboratories, and uniformity of methods both locally and globally would ensure a broad base of experience on which to base advice concerning fertilization needs for various crops on various soil types. The results from using such an extractant should have well established relations to the plant available portion of nutrients, *i.e.* to the crop response, to be of any practical use for plant producers. Extractants should be cheap, safe to use and easy to dispose of, and using as few extractants as possible for a complete assessments of relevant nutrient concentrations is desirable. Finally, a good soil extractant must be suitable for a wide range of soil properties, concerning texture, pH, organic content etc. (Houba et al., 1996;Jones Jr, 1998). As simple procedures as shaking samples with extractants may influence results, and standardization of such, presumed, minor issues would be important for comparability between laboratories. Even between laboratories using identical extractants and standardized methods, assessments of soil pH in the exact same samples have been found to deviate strongly, emphasizing further needs for standardization of protocols (Rayment, 1993).

Like extraction, also analysis of soil extracts may be done using numerous methods and techniques. Titration and colorimetric procedures have been much used historically, but since the 1990's, use of automated methods has increased rapidly, especially favoring the multi-elemental analysis methods Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES), also known as ICP-Atomic Emission Spectroscopy (ICP-AES), and – Mass Spectrometry (–MS). Atomic Absorption Spectroscopy (AAS) has also been used for a long time, and with newer instrumentation this method is effectively close to being multi-elemental as well. These methods will be reviewed more closely in section 3.4. Also automated colorimetric techniques, using either Segmented Flow Analysis (SFA) or Flow Injection Analysis (FIA), are commonly used and yield results rapidly, free from most soil matrix interferences and with a high sensitivity (Hettiarachchi and Gupta, 2008;Maynard et al., 2008;Ziadi and Sen Tran, 2008).

With the introduction of multi-elemental analytical techniques, the suggestion to replace the many different extractants with one single to analyze for all, or most, essential plant nutrients seems obvious. $CaCl_2$ has been proposed for determination of soil fertility as well as plant available concentrations of heavy metals (Houba et al., 1996). The Mehlich-3 extract, a mixture of acetic acid, ammonium nitrate, ammonium fluoride, nitric acid and EDTA, was developed also to extract essential plant nutrients, and good correlations to commonly used methods for soil analysis *e.g.* in Australian sugarcane production have been demonstrated (Ostatek-Boczynski and Lee-Steere, 2012). As a curiosity, Coca Cola® has been shown to work very well as an extractant for micronutrients (Fe, Cu, Zn and Mn) in soil. Specific advantages of Coca Cola® as compared to other extractants are the low price, the ubiquitous accessibility, even in the remotest parts of the world, high safety during handling and ease of disposal (Schnug et al., 1996).

Though new methods enable much more rapid analyses, in a few cases there are worries concerning the outcome. Colorimetric methods especially for P have been found to correlate poorly to ICP results, and no clear explanation for this has been found yet. It is speculated that colorimetric methods primarily measure ortho-phosphate, whereas the ICP measures both inorganic and organic P (Ziadi and Sen Tran, 2008).

An additional advantage of using a single extractant for all nutrients is that an overall picture of the soil fertility in a field is more likely to be obtained, as analyzing for a larger number of plant nutrients would only increase costs marginally. Plant nutrient antagonisms, meaning competition in uptake mechanisms in the rhizosphere or within the plant metabolism, can be discovered in a multi-elemental analysis. Antagonisms may lead to secondary nutrient deficiencies in plants, as *e.g.* severe Mg deficiency in tomato and kiwi caused by providing excessive levels of K in the growth medium. Reversely, too high levels of Mg can lead to severe K deficiency. In pastures, the Mg - K antagonism may cause grass tetany in cattle, a potentially lethal, acute Mg deficiency (Romheld and Kirkby, 2010). Also Mn uptake has been shown to interact with Mg uptake. Effects of high levels of K and Mn, however, are non-additive, indicating that all three ions do not compete for the same site (Heenan and Campbell, 1981). The form of N supply may affect uptake of other nutrients. The cations K, Ca, Mg and Na are taken up in higher amounts when NO_3-N is provided, an effect which is ascribed to NO_3^- acting as a counterion. For P, in the form of the anion PO_4^{3-}, the opposite effect occurs, as concentrations are lower when plants are provided NO_3-N compared to NH_4-N (Kurvits and Kirkby, 1980). It is, thus, evident that knowledge concerning levels and interactions of a number of nutrients is a prerequisite for efficient nutrient management.

2.1.4 Correlation to Plant Nutritional Status

The most severe drawback of the traditional soil analyses is the lack of correlation between extracted concentrations of nutrients using commonly applied methods and the actual plant available portion. As mentioned, many methods are developed specifically for use under certain soil conditions, why combining various soil types in an investigation may lead to a complete loss of relation between extracted nutrient concentrations and concentrations in the crop. This was demonstrated in a meta-study of trace elements, where the correlation between metal concentrations in plant leaves of various species and extracted concentrations in soil was poor or even non-existing (Menzies et al., 2007). For P, a lack of correlation is exemplified in Figure 4 by plotting early dry matter yield against Colwell P, the most common P extraction method in Australia. This method uses, like Olsen-P, 0.5M $NaHCO_3$ as the extractant (Mason et al., 2010).

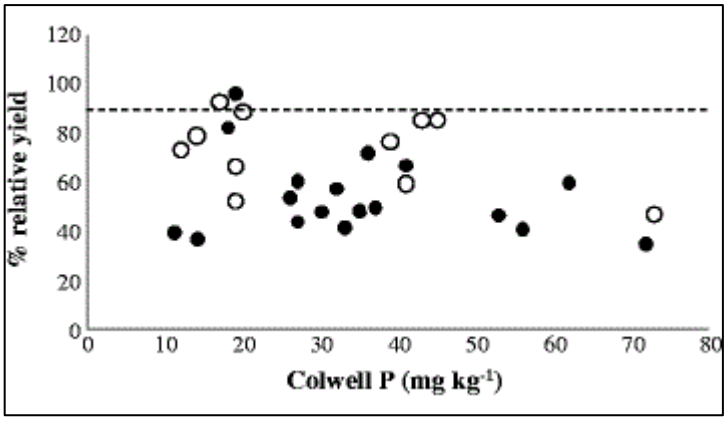

Figure 4. Dry matter yield of wheat at early stage, relative to a calculated maximum, plotted against Colwell P extraction results. From Mason et al. (2010).

No correlation was found at all between extracted P and early dry matter yield when collating data from different soils, as the amount of extracted P is highly dependent on soil characteristics as pH and soil contents of organic matter, clay and sand. This complicates the use of the results for general testing purposes (Bell et al., 2005;Debnath et al., 2010). In other words, the exact soil fertility is very often not assessed through traditional soil testing methods.

Despite the awareness of the challenges related to common soil analysis, a radical change has yet to come. Deciding on a single extractant is not delayed by lack of candidate extractants, rather because traditions play a major role, and one single extractant has not been able to outdo other. Standardization of traditional methods of soil analysis might not happen, as a new method using a completely different approach shows promising results regarding correlation to the plant accessible fraction of several nutrients.

2.1.5 Newest Developments in Soil Analysis

A very promising new method for soil analysis is the Diffusive Gradients in Thin films (DGT). It is still not used routinely in practical agriculture, but it is widely used in research areas such as geochemistry, water quality monitoring and assessment of plant available nutrients in water and soil (Davison and Zhang, 2012). The DGT consists of an ion-exchange resin gel covered by an ion-permeable, diffusive, gel membrane and a protective filter, which separates the resin from the bulk solution, *e.g.* soil. The DGT is sketched in Figure 5. As long as the resin is not saturated, it functions in principle as an infinite sink, and the concentration at the resin gel surface is maintained around zero. This leads to the formation of a concentration gradient through the diffusive layer, C_{DGT}, and of a depletion zone mimicking that of the rhizosphere. The DGT, hence, mimics plant uptake of nutrients limited by diffusional supply, such as P, Cu and Zn (Tandy et al., 2011).

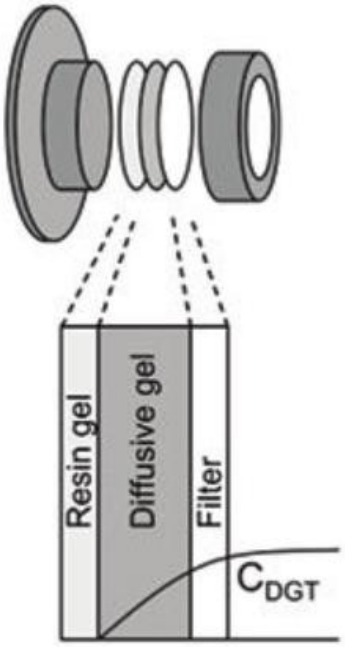

Figure 5. Overview of the components in the DGT. C_{DGT} is the concentration gradient of a given nutrient from the soil solution into the DGT. From Mundus et al. (2011).

After a given deployment time, the ions in the resin gel are eluted, and the nutrient concentration is determined. Deployment time may vary from 1 hour and up to as much as 3 months in aqueous solutions. In soil, deployment times from hours and up to a few days are most common (Zhang and Davison, 1995;Nowack et al., 2004;Tandy et al., 2012). In pot trials, nutrient concentrations determined by DGT have been shown to estimate plant available concentrations of Cu, Zn, P and the contaminant Pb very well (Tandy et al., 2011;Agbenin and Welp, 2012). Arsenic availability was successfully determined in another greenhouse experiment, showing strong potential for assessment of the risk of cultivating polluted soils (Cattani et al., 2009). Promising results have also been obtained for measuring plant available K concentrations, though further improvement of this method is still needed (Tandy et al., 2012). An extensive field study in Australia documented the DGT accuracy in assessment of plant available P for wheat cultivated in a wide variety of soils (Mason et al., 2010). Thus, DGT has proven effective for assessment of plant available concentrations of a number of nutrients in soil, and testing and refining of the methodology takes place at a high pace just now, judging from the number of peer-reviewed papers from the last 2-3 years. However, problems still reside with certain nutrients, as *e.g.* Mn where plant availability depends on redox state. Only in anaerobic conditions, where Mn is in the plant available form of Mn(II), an acceptable correlation was obtained between DGT measurements and plant tissue concentrations. What would be really useful is a method able to handle both aerobic and anaerobic conditions (Mundus et al., 2012).

Visual, Near Infrared and Mid Infrared spectroscopy (Vis, NIR and MIR) have been introduced successfully for determination of several soil characteristics. Visual light is defined as light of wavelengths between 400 and 700nm, NIR is light of wavelengths between 700 and 2500nm, and MIR light has wavelengths between 2500 and 50,000nm. The reflectance or transmittance of Vis, NIR and MIR light contains information about the molecular composition and particle size

distribution of a sample, as molecular movements interact with the light. There is no direct information about atomic concentrations in the light spectra, why parameters that have been predicted successfully using these methods are mainly physical, not chemical. Vis-NIR has resulted in predictions with high accuracy of CEC, sand, silt, clay and total C and N, which are correlated to other soil constituents, with success, whereas predictions of specific nutrient concentrations were largely unsuccessful (Chang et al., 2001;Sorensen and Dalsgaard, 2005). Comparisons of prediction performance of Vis-NIR and MIR have concluded that MIR is superior for pH, CEC, clay, sand, silt and organic C. This may be explained by the higher intensity and specificity of the signal in the MIR area as compared to NIR (Pirie et al., 2005;Rossel et al., 2006). The major advantages of spectroscopic soil analysis are the potential use in situ and the fact that several properties can be determined from one measurement, *i.e.* one spectrum. Compared to laboratory analysis, there is a significant reduction in costs once the equipment has been acquired. No extractants are needed, and handling is completely safe (Du and Zhou, 2008;Nduwamungu et al., 2009;Du and Zhou, 2011).

2.2 Plant Elemental Analysis

Plant elemental analysis traditionally provides knowledge about the nutrient concentration of a plant. The concentration of nutrients in a given plant part may provide a measure of the plant nutritional status up front, and the question of plant availability of nutrients in the soil is circumvented (Parks et al., 2012). In other words, “the patient” is examined rather than the environment of it. Acute disorders as well as, in some cases, latent disorders may be revealed by gauging plant material, whereas this is not possible using soil analysis. If a specific disorder is suspected, plant analysis may, hence, bring about the “proof”.

In Denmark, the use of plant analysis in horticultural production is much more common than in agriculture (M. Bojesen, personal communication, 2013). As mentioned in section 0, soil analysis will often be useless for woody and perennial species, but another reason for the more widespread use of plant analysis in horticulture may be that secondary deficiencies are more prevalent here. Secondary deficiencies are caused by inadequate translocation of nutrients to plant organs, rather than actual nutrient shortages in the growth medium. Examples are blossom end rot in tomato and bell pepper, tipburn in Chinese cabbage and bitter pit in apple. Analyzing for nutrient concentrations in specific plant parts can be of assistance to avoid these disorders. The concentrations of nutrients in plant material in pasture and other forage crops are also commonly analyzed, as these are of major importance to animal nutrition (Römheld, 2012). One barrier to the increased use of plant analysis is the price versus the perceived value by farmers (L. Knudsen, personal communication, 2012). From the laboratories, much has been done to increase the value of plant analysis, and it is now possible to obtain results from a plant analysis in some cases already the same day as the laboratory receives the plant sample. Previously a processing time of up to two weeks could be found, potentially causing major yield losses if any action should have been taken. Prices are still relatively high compared to soil analysis but have declined significantly during later years. This has caused the use of plant analysis to increase slightly in recent years. Plant elemental analysis covers a range of methods analyzing the nutrient status of specific parts of crops. In practical agriculture, ICP-OES is widely used, and also AAS is common. The more sensitive ICP-MS is predominantly used in science, as the increased sensitivity is superfluous for most agricultural applications, it is more complicated to operate and

both acquisition and running costs are significantly higher. Section 3 describes and discusses the various analytical techniques in more detail.

The relation between plant availability of a given nutrient and yield or biomass production of the plant is generally described by the Mitscherlich curve (Figure 6).

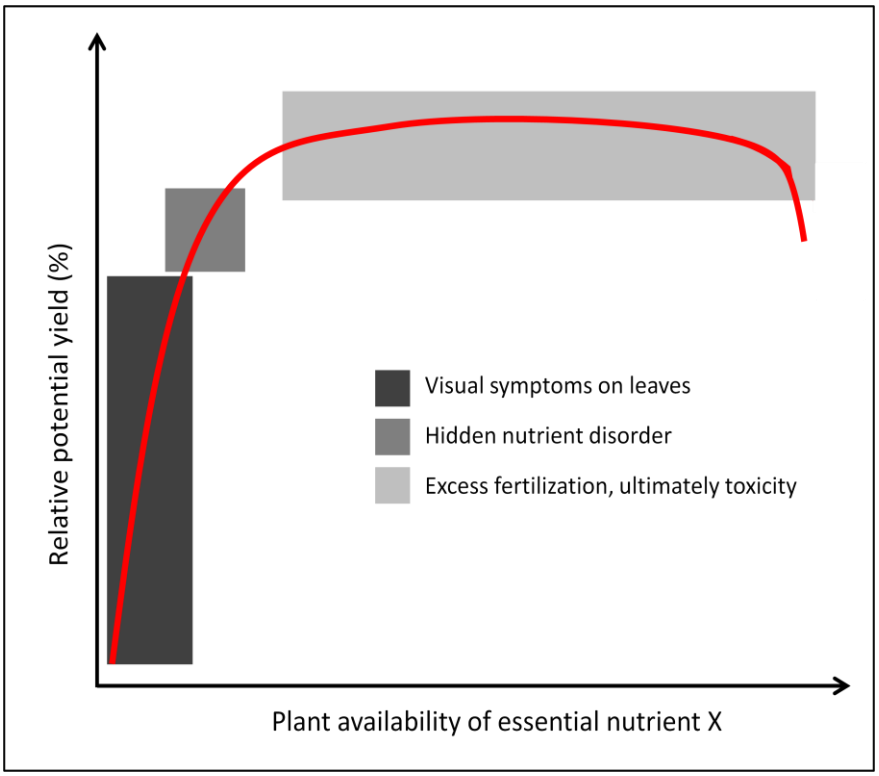

Figure 6. Mitscherlich curve showing relative, potential yield as a function of plant availability of a given nutrient. The dark grey rectangle indicates severe deficiency of the nutrient, resulting in visual leaf symptoms. The medium grey square indicates the area of hidden or latent disorder, where yield is reduced, but no visual leaf symptoms occur. The light grey rectangle indicates ample and excessive supply, ultimately resulting in reduced yields due to toxicity.

At very low levels of plant availability of an essential nutrient, the plant will develop visual leaf symptoms, enabling diagnosis of the disorder by simple inspection, indicated by the dark grey rectangle in Figure 6. The light grey area shows excessive fertilization, ultimately resulting in toxicity. Providing nutrients in an amount resulting in the highest possible yield without over-fertilizing is extremely difficult. Often a slight over-fertilization or hidden nutrient deficiency (medium grey square in Figure 6) occurs even in well-managed farmlands; situations that the plant producer is in effect unable to discover without the assistance of advanced, analytical methods.

Slight variations in the shape of the Mitscherlich curve may occur when analyzing specific plant parts or focusing on specialized situations (Reuter et al., 1997). One of the most notable of these is the Piper-Steenbjerg effect, depicted in Figure 7.

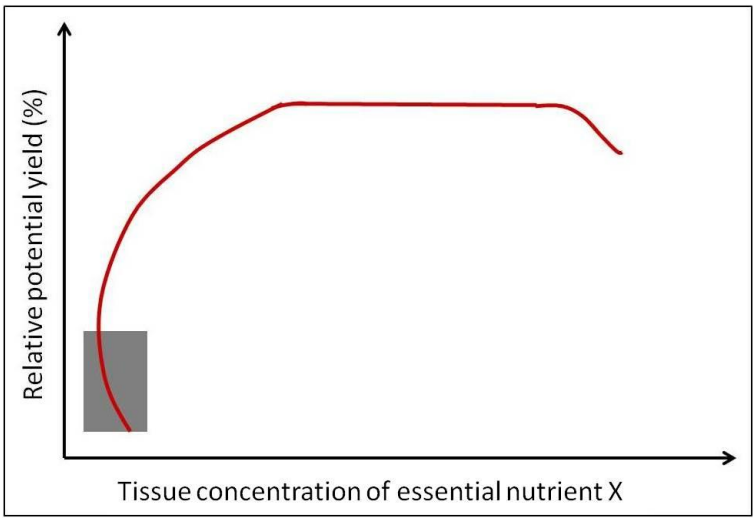

Figure 7. Mitscherlich curve including a Piper-Steenbjerg effect at the lowest yields, indicated by the grey rectangle. After Smith (1962).

At very low nutrient concentrations in plant tissue, a transient, negative correlation between plant yield and plant nutrient concentration, called the Piper-Steenbjerg, effect may arise. It is caused by an incongruity between biomass production and uptake rate, leading to a net drop in tissue concentration (Wikstrom, 1994).

2.2.1 Critical Concentration Thresholds

Plant elemental analysis is traditionally carried out as an analysis of the concentration of one or several nutrients in a specific plant part. This value is compared to a table of critical concentration thresholds or, more commonly, sufficiency ranges to determine whether the crop is adequately supplied or fertilization is necessary. The critical concentration threshold is defined as the lowest concentration of a nutrient required for optimal growth and maturation (Ulrich, 1952), and the sufficiency range is simply the concentration range at which plants are adequately supplied. Much effort has been put into defining critical concentration thresholds or sufficiency ranges of essential nutrients for all the different cultivated crops, and the results can be found in large tables as for instance in Reuter et al. (1997). Values are consistently re-evaluated, and assisting parameters for improved correlation to plant nutritional health are developed.

Especially for macronutrients, good correlations are found between plant concentrations and plant nutritional status, typically measured by yield or biomass. One example of this is provided in a series of experiments concerning S concentrations in corn and sugar beet and their relation to final yields. Though crops were cultivated on a large variety of soils, S concentrations correlated very well to yields, along a Mitscherlich curve (Figure 8). It was furthermore possible to determine a common critical concentration threshold for each species (Hoffmann et al., 2004;Pagani and Echeverria, 2011). Combining total S concentrations with the N:S ratio has been suggested to increase the power for assessing nutritional status, and thereby fertilizer needs, during vegetative growth of wheat (Reussi et al., 2011), however, no improved results were obtained by including this ratio in an analysis of corn (Hoffmann et al., 2004).

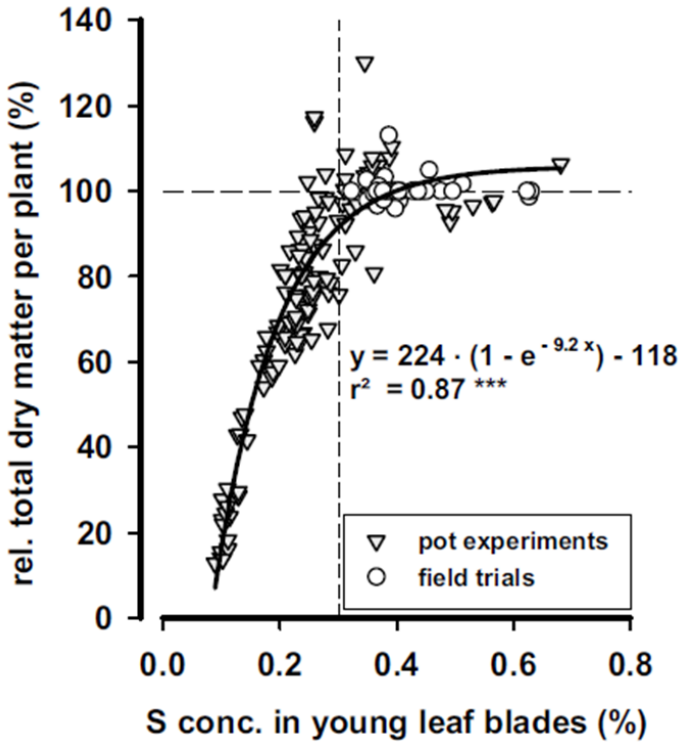

Figure 8. Relative total dry matter per sugar beet plant at harvest versus S concentration in young leaf blades. From Hoffmann et al. (2004).

Factors such as species, genotype, plant age and plant part influence critical concentration thresholds, very often to significant degrees (Lewis et al., 1993;Nabi et al., 2006). General differences in nutrient requirements may be found between groups of plants. For example, dicotyledonous species need significantly higher Ca concentrations than monocots to obtain maximal growth rates. This is illustrated in Figure 9, using tomato and ryegrass as representatives for the two classes.

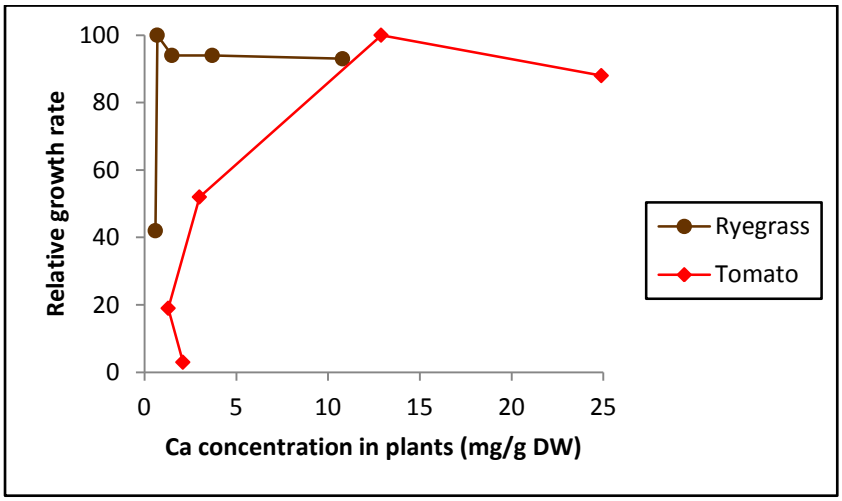

Figure 9. Relative growth rate plotted against plant Ca concentration. Data derive from Loneragan et al. (1968) and Loneragan and Snowball (1969).

Due to these clear variations in nutrient needs, threshold values or sufficiency ranges are specified for each plant species. It is also commonly indicated to which plant part the values

apply. In wheat, Zn concentration thresholds have been shown to vary by more than 300% between plant parts, from 10.5mg/kg dry weight (DW) in the stem up to 34.1mg/kg DW in the ear, making such specifications absolutely essential (Dang et al., 1993). The differences between concentrations in various plant parts are relatively consistent at adequate Zn levels, as shown in Figure 10.

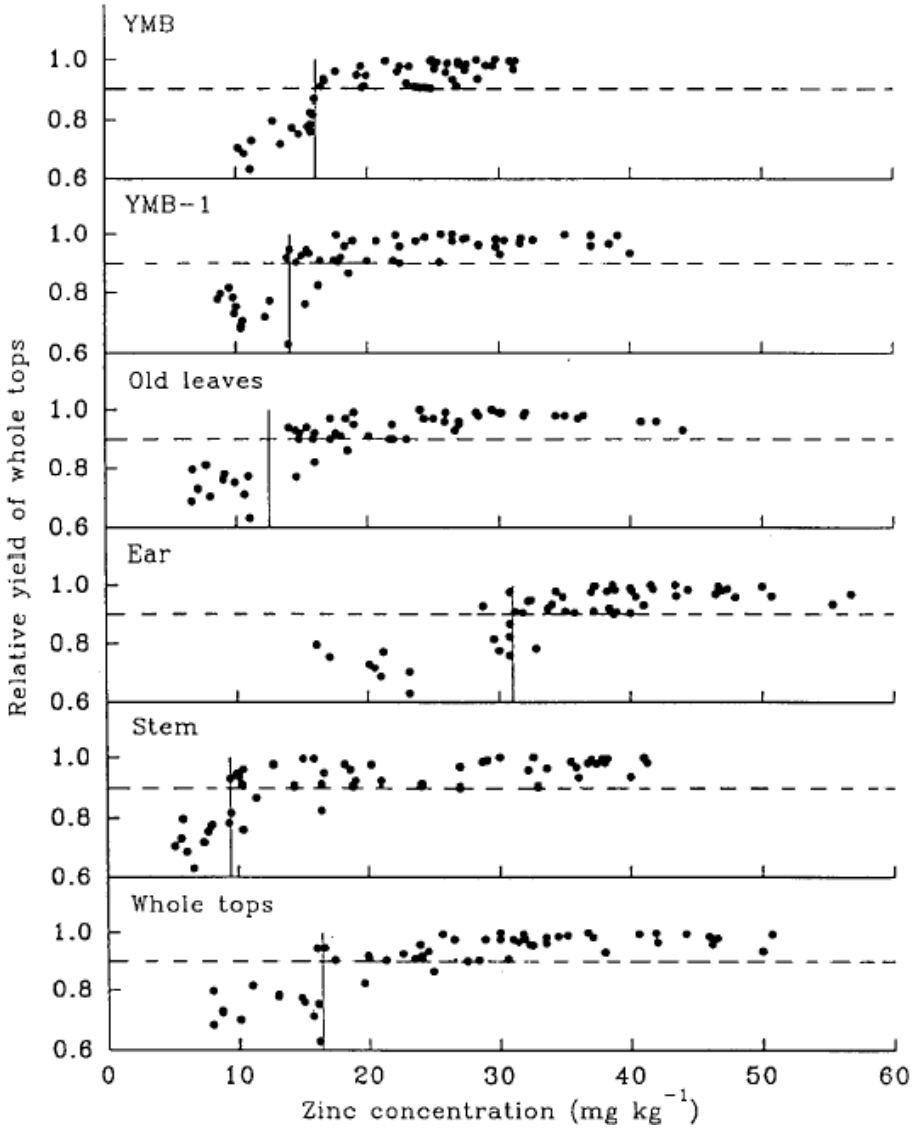

Figure 10. Relative whole top biomass yield plotted against Zn concentrations in different plant tissues of wheat. Calculated critical Zn concentrations are indicated by vertical lines. Data from Dang et al. (1993)

The physiological age of a plant or plant part affects nutrient concentrations to a considerable degree; after nutrient supply this is the single factor affecting nutrient concentrations the most (Römheld, 2012). As plants approach maturity, the nutrient demand for new growth declines, why critical deficiency thresholds on a whole-plant level decrease for most nutrients, with Ca, B and Mn as the exceptions (Hill et al., 1979;Römheld, 2012). A simple dilution effect can also occur due to the increased total biomass. The relation between age and critical deficiency thresholds has been demonstrated by Reuter et al. (1981) (Figure 11).

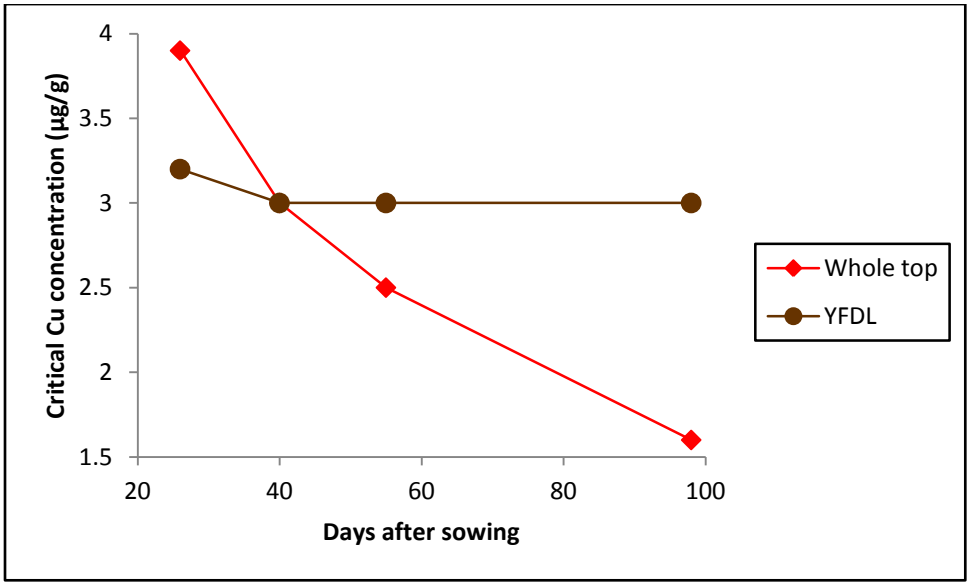

Figure 11. Critical Cu concentrations (µg/g) in subterranean clover as a function of plant age. Data derived from Reuter et al. (1981).

It is clearly seen in the figure, that where the critical Cu concentration declines rapidly as the plant ages, the youngest, fully developed leaf (YFDL) has an almost stable value throughout the growing period. For nutrients of low phloem mobility, selecting the YFDL for analysis is therefore an obvious choice. Concentrations of nutrients with high phloem mobility may show less variability in the YFDL due to remobilization. However, concentrations in older leaves will also be affected by the remobilization, and with increasing leaf age also comes an increasing risk of leaf damage caused by biotic or abiotic stresses. Therefore, the YFDL is the common, and general, choice of plant part for analysis.

Not only factors within the plant affect critical concentration thresholds; also soil characteristics and climatic conditions may be of importance. During sampling of plant material, even air and soil temperatures, plant turgidity and time of the day may influence the results. In perennial crops also seasonal fluctuations occur, though these may be handled by proper sampling procedures. For micronutrients, concentrations are often so low that using concentration thresholds can be very difficult. Minor measuring errors or effects from *e.g.* genotype or climate that have not been accounted for may lead to false conclusions. In a study in wheat and cotton, no significant differences between Cu concentrations in leaves of Cu deficient and Cu sufficient plants could be found (Rao and Ownby, 1993). Supplementing total nutrient analyses with other diagnostic methods when possible can therefore be a great advantage, especially for micronutrients. Manganese deficiency is easily diagnosed by measuring the Fv/Fm value of chlorophyll fluorescence. Relating Fv/Fm values to total Mn concentrations in youngest, fully developed leaves however is not strictly linear, supporting the use of a method based on physiological functionality rather than on simple concentrations (Schmidt et al., 2013). Such alternative methods are unfortunately not available for all micronutrients at present.

In practice, plant analysis today is used to confirm, or disprove, suspicion of nutrient disorders, and in case of poor growth, it will typically be part of an investigation of possible reasons. Routine plant analysis used as a guidance tool for fertilization is less common, but as costs

decrease and awareness of the advantages increases, the use of plant analysis in practical agriculture is likely to intensify.

2.2.2 The Diagnosis and Recommendation Integrated System

The Diagnosis and Recommendation Integrated System (DRIS) has a different approach to assessing fertilizer needs than using critical concentration thresholds or sufficiency ranges alone. DRIS is based on ratios between nutrient concentrations, in its most advanced form even including interactions with and between soil composition, farming practices and any other yield influencing variables about which information can be obtained. The calculated indices are related to yield, thereby developing a complicated system to assess whether crops are well balanced with respect to nutrients and possibly also other factors (Sumner and Beaufils, 1975). Due to the use of ratios, the DRIS to some extent avoids the problem of critical concentration thresholds being interdependent. The indices have been shown to be generally applicable for a given plant species, irrespective of age or time of sampling. No differentiation of indices in relation to cultivars was necessary in cotton (Singh et al., 2012), but ratios including P concentrations were found to differ highly between wheat genotypes (Yaseen and Malhi, 2009). Also the region of cultivation has been found to influence DRIS (Römheld, 2012).

The disadvantage of DRIS is first of all the vast data collection necessary; both during development of the system and when using it. This makes it relevant mainly for high value crops, such as tree fruits, or other perennial cropping systems, *e.g.* sugar cane, where a crop is cultivated for several years in the same field (Sumner and Beaufils, 1975;Raghupathi et al., 2004;Raj and Rao, 2006;Srivastava and Singh, 2008).

2.2.3 Plant Ionomics

As advances in analytical chemistry have made plant analysis much faster and more exact, new perceptions on how to use the results arise. The traditional view based on the “Law of the Minimum”, where one nutrient is considered at a time, has been challenged repeatedly since the late 1980’s (Ingestad, 1987;Parent and Dafir, 1992;Parent et al., 2013), and where DRIS is an attempt to include elemental interactions in a diagnosis system, awareness that this might not be a satisfactory approach is rising (Parent et al., 2013).

The ionome of a plant or plant part consists of all the elements contained in it, including essential, beneficial and, in some definitions, even toxic elements (Salt et al., 2008;Baxter, 2009). The elemental homeostasis of the plant ionome is controlled by a huge network of interactions between the different elements, a subset of which is presented graphically in Figure 12 (Baxter, 2009). Experiments that focus exclusively on one or a selected few elements will not be able to reveal any of these interactions, why diagnostic systems derived from these results will be sensitive to alterations in the factors previously discussed. Predicting elemental interactions in this giant network based on theoretical assumptions will in most cases not be possible based on the knowledge we have today, as the majority of the genes involved in ionome regulation are still unknown. The exception is elements that are chemically alike and therefore able to compete for uptake and transportation mechanisms (Baxter, 2009;Singh et al., 2013). Instead the elemental interactions must be determined experimentally; an approach which is becoming more feasible with the developments in multi-elemental analytical methods as well as easily accessible programs for multivariate data analysis.

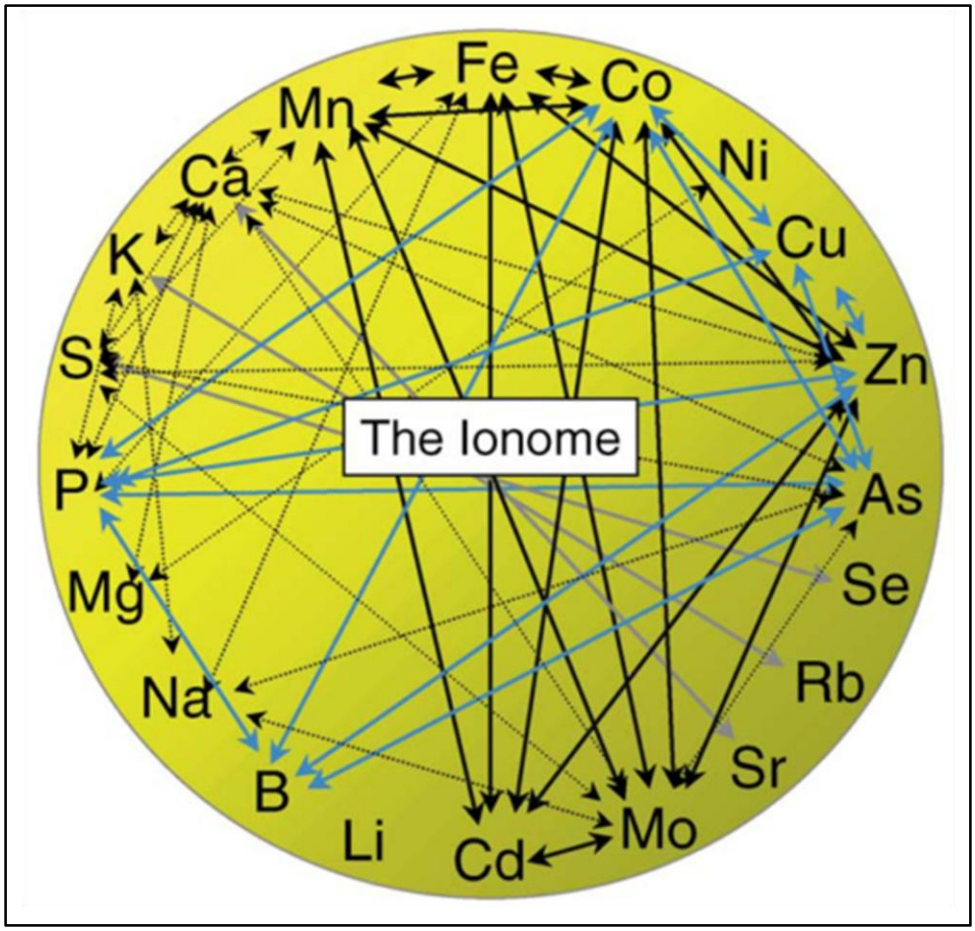

Figure 12. Genetic, physiological and chemical interactions between elements; essential, beneficial and toxic elements are included. Only a subset of known elemental interactions is presented in this figure, and unknown interactions are likely to exist as well. From Baxter (2009).

Recently it has been speculated that including concentrations of a large number of nutrients may not even be enough to obtain the best overview of plant nutritional status. The raw concentration data are biased, as they heavily depend on scale and on the denominator for nutrient concentrations, *i.e.* for instance fresh or dry matter. If any of these change, the conclusions of an ionomic analysis may do the same. By mathematical transformation of the raw data or correlations between them, scale-independent data material results, and more stable conclusions can be drawn (Parent et al., 2013). It is further suggested that the figure of a barrel filled with water, commonly related to the Law of the Minimum, be changed into a mobile, as shown in Figure 13.

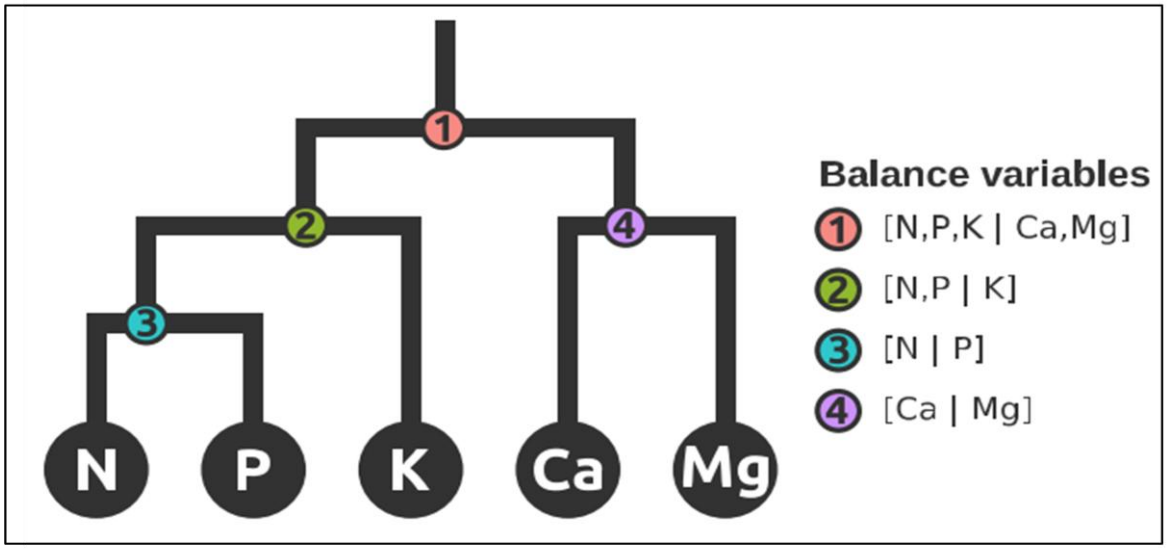

Figure 13. A Mobile at mass equilibration point illustrates four hierarchically nested balances that represent a subspace of nutrients in the ionome. From Parent et al. (2013).

According to this proposition, inter-dependent nutrients as *e.g.* K and Ca should be somehow connected, meaning that they are placed on either side of a balance point. The mobile concept captures nutrient interactions and adjusts nutrient balances according to changes in single nutrients, thereby providing an overview of the whole plant ionome. Imbalances will be apparent, as the mobile will be unbalanced at higher or lower levels (Parent et al., 2013).

An overview of the plant ionome will enable diagnosis of physiological and biochemical changes within the plant, and using ionomics, biomarkers for nutrient imbalances as well as other biotic and abiotic stresses may be found. In addition, it may serve as a cheaper and faster method for phenotyping mutants compared to genome sequencing. With the rapid developments in multi-elemental analytical equipment, this is a research area that attracts growing attention (Salt et al., 2008).

3 Methods of Plant Elemental Analysis

Since the concepts of plant nutrition were founded, much effort has been put into developing methods for diagnosing nutritional disorders. As discussed, this is usually done by determining the nutrient concentrations in plants or plant parts, but alternative methods based on secondary indices are increasingly gaining foothold. At present, methods for fast, spectroscopic analysis are being developed to provide rapid (from a few minutes down to immediate), cost-efficient (in some cases effectively no cost per sample after acquiring the equipment) and accurate analyses at early stages of nutrient deficiencies. The following is a brief review of methods used historically in science and agriculture followed by state-of-the-art techniques for determination of total nutrient concentrations in plants. Finally a thorough introduction to the newest methods for fast, spectroscopic analysis is given.

3.1 Visual Inspection

A pronounced deficiency of any essential plant nutrient causes specific, visual symptoms, which are recognizable by a trained person. The identification of these symptoms is a very basic and straight-forward competence for any plant producer or plant scientist. It can be done directly in the field, without using any equipment, completely independent from laboratories and hence, at no cost.

The appearance of visible symptoms of a nutrient deficiency is associated with the physiological function of the nutrient. All nutrients have several functions in plant metabolism, but the dominating function typically expresses the strongest phenotype during deficiency, and leads to the first, visual symptoms. An example of pronounced Cu deficiency, “white tip disease”, is shown in Figure 14. Copper is essential for the lignin biosynthesis, why a pronounced deficiency in the vegetative phase leads to wilting of leaf tips in cereals, as cell walls collapse (van Maarschalkerweerd et al., 2013).

Figure 14. Pronounced Cu deficiency in barley leaf (bottom), compared to healthy control plant (top).From van Maarschalkerweerd et al. (2013).

Stratification of the symptoms relates to phloem mobility. Nutrients with high phloem mobility are re-translocated from the oldest leaves to the youngest in case of a deficiency, resulting in visible leaf symptoms appearing first in the older leaves. Deficiency symptoms of nutrients with low phloem mobility generally occur first in the newest growth, which in this way characterizes the “present” nutrient availability situation. An overview of phloem mobility of the essential nutrients is found in Table 1. Generally, the macronutrients, with the exception of Ca and S, are highly mobile in the phloem. A concentration of only a few µM Ca in phloem sap would cause clogging of the sieve plates due to increased callose formation and subsequent swelling, why the concentration must be kept low (White, 2012). This phloem immobility of Ca is what makes it a major trigger of secondary deficiencies, as transportation into non-transpiring tissues with the xylem stream is low. Visual S deficiency symptoms occur first in new growth, indicating phloem immobility, but it has been demonstrated that during development of new leaves at adequate S supply, soluble S is extensively remobilized from mature leaves (Anderson, 1996). Sulphur is therefore classified as an intermediately phloem mobile nutrient. Micronutrients are considered at the least intermediately phloem mobile, with the exceptions of Mn and B. Boron is usually phloem immobile, but in sorbitol-producing species of *Pyrus*, *Malus* and *Prunus* genera, B has been shown to have high phloem mobility. This has been suggested to be due to the formation and transportation of stable B-sorbitol complexes *in planta* (Brown and Hu, 1996). Boron is therefore placed in two categories in Table 1. In some cases, Ni has also been found to be highly phloem mobile. The exact mechanisms causing these differences between mobility of micronutrients have not yet been described (Page and Feller, 2005;Riesen and Feller, 2005).

Table 1. Phloem mobility of essential elements. From (Anderson, 1996;Brown and Hu, 1996;Page and Feller, 2005;Riesen and Feller, 2005;White, 2012).

Phloem Mobility		
High	**Intermediate**	**Low**
N (amino-N)	S	Ca
P	Fe	Mn
K	Cu	B
Mg	Mo	
Cl	Zn	
[B]	Ni	

A number of situations may blur an otherwise straightforward, visual diagnosis of nutrient deficiencies. If multiple deficiencies occur simultaneously, one symptom may dominate over another, leaving the latter undetected, or a mixture of symptoms occurs. In other situations, nutrients may be interdependent. For example, Mo is a constituent of both nitrate reductase, required for assimilation of soil nitrate, and nitrogenase, which is essential for symbiotic N fixation. Thus, Mo deficiency will result in visual symptoms of N deficiency, though sufficient amounts of N may be available to the plant (Skarpa et al., 2013). Finally, visual symptoms of deficiency will often be influenced by, and sometimes confused with, varying degrees of other biotic and abiotic stresses such as drought, flooding, insect attacks or diseases (Grundon et al., 1997).

As a basic method for low-income smallholders, visual detection is the main tool to assist in management of the often sparse input resources available. However, the late appearance of distinct, visible symptoms may cause substantial yield losses, as it will often be too late to correct the problem within the same growing season. Thus, visual diagnosis is insufficient for supporting nutrient management in intensive, modern agriculture.

3.2 Gravimetric and Colorimetric Analysis

Gravimetric and colorimetric analyses were among the first methods to be developed for determination of nutrient concentrations in plant tissue. Both types are single-element methods, determining one element at a time. In gravimetric analysis, the mass of an analyte or a derivative of the analyte is determined. An example is the conversion of sulphur into sulphate through combustion, followed by precipitation of the sulphate as barium-sulphate. The precipitate is dried and weighed, and the sulphur concentration in the original material is calculated on the basis of this. The gravimetric methods are rarely used today, as already some 80 years ago, the methods were known to be inconvenient and very often imprecise, even in experienced hands (Piper, 1944).

In colorimetric analysis, a chemical reaction is carried out between the analyte and a reactant, yielding a colored substance, a chromophore, either directly or by one or more subsequent reactions. The resulting color is compared to standard solutions or determined using a spectrophotometer and plotted on a dilution curve (Bromfield, 1987;Anjos et al., 2009). A commonly used colorimetric method is the determination of nitrate by reduction to nitrite followed by conversion to nitrous acid, which triggers reactions leading to the synthetization of a red-violet azo dye (Alves et al., 2000). Colorimetric methodologies are implemented in simple

test strips developed for a large number of plant nutrients including N, S, K, Mg, Cu and Fe, each in one or more molecular organizations (Millipore, 2013). These strips are for use in water or solutions, limiting the use in plant analysis, but they do offer a fast, in-situ and low cost method for semi-quantitative assessment of nutrient concentrations in turgid crops. In potato and leafy vegetable crops, K^+ and NO_3^- are commonly analyzed for using test strips in plant sap. This is collected by pressing petioles manually or using simple equipment directly after sampling, or alternatively by homogenizing and filtering petioles and in some cases whole or part of leaves (Errebhi et al., 1998;He et al., 1998;Bantan et al., 1999;Hartz, 2007;Parks et al., 2012).

For both gravimetric and colorimetric methods, it is crucial to be aware of the specificity of the methods, and whether the complete amount of analyte takes part in the chemical reactions, as residuals are unlikely to be detected. For colorimetric methods, the sensitivity may not always suffice, especially considering nutrients in low concentrations (Chapman et al., 1996).

3.3 Enzyme Assays

A number of correlations have been found between nutrient deficiencies and activities of specific enzymes, and test kits to use in the field or on the farm have been developed (Barakiva and Lavon, 1968; 1969;Barakiva et al., 1969). The enzymatic tests are carried out by infiltrating a leaf disc or a processed leaf solution with a solution containing the nutrient in question and monitoring the change in enzyme activity afterwards. A deficient plant sample will show increased activity, as the missing nutrient is provided. Though apparently a simple technique, the procedure described by Barakiva and Lavon (1969) includes maceration, filtration and several turns of centrifuging with addition of various substrates in between each, which decreases the convenience of the method substantially. Delhaize et al. (1982) developed a field test for detecting Cu deficiency in subterranean clover, but this method also suffers from being time consuming as well as complicated to operate. Supporting this conclusion, the bulk of literature on enzyme assays derives from the late 1960's to the beginning of the 1980's, and no enzymatic test kits are, to the knowledge of the authors, commonly used for diagnosing plant nutritional disorders today.

3.4 Atomic Spectroscopy

The most frequently used methods for plant analysis are at present based on atomic spectroscopic analysis. A vast number of methods are found in this field, with numerous variations and combinations. Here, an overview of the most commonly used methods, Flame ionization – AAS (F-AAS), Graphite Furnace – AAS (GF-AAS), ICP-MS and ICP-OES, is given.

Atomic spectroscopy aims at detecting the exact, atomic concentrations in a sample. The actual analysis is swift, and multi-elemental measurements may be obtained by ICP-OES and –MS. Atomic absorption spectroscopy is single-elemental, but newer instruments provide rapid shifts between detected elements, meaning that multi-elemental performance is approached. In general terms, the techniques consist of three steps: Digestion, ionization and detection. During sample digestion, dry, ground sample material is broken down into inorganic, soluble ions. This can be tedious and introduce error into the measurements, why mastering this step is just as important as the analysis itself. Digestion is followed by a complete ionization, which allows sample introduction into the analytical instrument. Finally, elements are detected based on either light absorption (AAS), light emission (-OES) or mass to charge ratios (-MS).

There are major differences between the atomic spectroscopic techniques in accuracies, detection limits, costs and required skills for operation. A brief comparison of the methods is given in Table 2. For a plant producer, they are all methods that require the assistance of a laboratory. This means relatively high costs per analyzed sample, and in case of an acute nutrient disorder, precious time may be lost during transport, handling and analysis of samples. Nevertheless, as discussed in section 2.2, plant analysis using these techniques is employed and promoted in modern agriculture for fertilizer optimization purposes, due to the high accuracy of the results, which relate to present nutrient status of the crop.

Table 2. Schematic, generalized comparison of F-AAS, GF-AAS, ICP-OES and ICP-MS (Mermet and Poussel, 1995;Thermo, 2013). Detection limits are displayed for Ni+, the least abundant essential element in plants. Huge variation in these figures can be found in literature, why these are merely indications of magnitude.

	F-AAS	**GF-AAS**	**ICP-OES**	**ICP-MS**
Multi-/single element	Single	Single	Multi	Multi
Acquisition costs	1x	2x	3x	5-10x
Running costs	Low	Medium	High	Highest
Sample input	Digested liquid	Solid, slurry or liquid	Digested liquid	Digested liquid
Dynamic range	10^3	10^2	10^6	10^8
Detection limit (Ni^+, ppb)	100	0.1	5	0.001

3.4.1 Sample Digestion

To prepare samples for introduction into atomic spectrometers, they have to be digested. The digestion process degrades organic material, which can block the sample introduction system, and results in a sample containing only inorganic, soluble ions. Any residual organic C may cause analytical problems such as plasma instability (in ICP instruments) and polyatomic interferences, why an efficient digestion process is of utmost importance (Husted et al., 2011).

Digestion may be carried out as wet digestion or dry ashing, also called combustion. In wet digestion, samples are digested in an oxidizing and acidic environment. A strong acid, often HNO_3, works as an oxidant, sometimes in combination with H_2O_2. The latter is only a weak oxidant, but it is able to regenerate HNO_3 from NO_X and thereby increase the overall efficiency of the digestion. Both open and closed vessel methods exist. Open vessels are most prone to contamination and loss of analyte, why they are unsuitable for trace analysis. However, they allow easy high-throughput, and equipment is relatively low cost, which are great advantages if a sufficient quality of the digested sample is obtained. The closed vessel methods provide efficient digestion, as samples in an acid, oxidizing environment are heated under pressure, leading to a higher boiling point. A common workflow for this is to mix dried, finely ground plant material with H_2O_2 and HNO_3. The mixture is heated under pressure, *e.g.* up to 140°C, in a microwave and, after cooling, diluted to an appropriate acid concentration, according to spectrometer details (Flores et al., 2007;Hansen et al., 2009). Recently, new closed vessel, wet digestion methods have been developed enabling high-throughput digestion of small sample sizes, 1 - 20mg dry weight plant material, which is down to 4‰ of the amount needed for common macroscale digestion (Hansen et al., 2009). This is extremely useful in many scientific applications, where sample material may be limited.

Dry ashing is the method of choice when digesting samples containing high amounts of carbon, such as coal and graphite, or much glycerol, which is used *e.g.* for stabilization of chloroplasts isolated from tissue and cell fractions, as combining HNO_3 and glycerol in wet digestion constitutes a serious safety risk (Flores et al., 2007;Husted et al., 2011). The decomposition of organic material in dry ashing is based on a reaction between the organic material and oxygen at high temperatures, or in some cases excited oxygen or oxygen radicals in plasma (Flores et al., 2007). Oxygen is considered analytically pure, why dry ashing has a very low risk of contamination as compared to wet digestion, where strong acids are added to the sample. However, if open vessels are used, there is a high risk of analyte loss due to volatilization as well as of cross-contamination. Closed vessel procedures for dry ashing have been developed to limit this risk, but these systems do not have the capacity for high-throughput. Together with recent advances in wet digestion this means that dry ashing is presently used only to a limited extent (Flores et al., 2007;Husted et al., 2011).

3.4.2 Sample Introduction and Ionization

Atomic Absorption Spectroscopy

For AAS, samples are ionized either by flame ionization or by using a GF (F-AAS and GF-AAS, respectively). In flame ionization, the liquid, digested sample is sucked into a flame with a temperature of 2,000-3,000K. The liquid evaporates, whereas the solids are ionized, and their concentration is determined (Harris, 2007). A GF is an electrothermal atomizer in the form of a graphite tube in which the sample is placed and heated. Depending on sample and instrument, the temperature of the tube may reach up to 3,000 – 3,300K (Bucksci, 2013;PerkinElmer, 2013). Liquid, slurry or solid sample material can be placed in the graphite tube without any prior digestion step, which is a great advantage of the system. Avoiding the digestion step means avoiding a major source of error in the form of sample loss or contamination, as well as a significant reduction of time consumption. However, measuring on solid material is complicated, and the rate of success is highly dependent on the specific matrix and the desired elements, why liquid samples are the most common, especially when connected to AAS (Rodrigues et al., 2007;Resano et al., 2008). For analysis of plant material, GF is most frequently used for determination of concentrations of heavy metals and contaminants such as Pb, Ni, Cu and Cd, and samples are often extracted or digested before sampling into the GF. (Jorhem et al., 2000;Liao et al., 2000;Sun et al., 2008;Araujo et al., 2009).

Inductively Coupled Plasma

For –MS and –OES instruments, sample ionization is usually done by ICP. The first step in this procedure is the formation of an aerosol from the liquid, digested sample using a nebulizer. The aerosol is led into a spray chamber, in which the larger droplets are removed. The resulting fine mist, constituting as little as 1-2% of the original amount of sample pumped into the nebulizer, then continues further into the plasma (Browner and Boorn, 1984).

Two types of nebulizers exist; pneumatic and ultrasound. Pneumatic nebulizers come in numerous designs, which vary in their ability to handle small sample sizes and solids in the sample, in resistance to strong acids or caustic, in gas pressure needed and in obtained precision and detection limits. The most common design is the concentric, pneumatic nebulizer. It works much like a spray deodorant using the mechanical force of a gas flow, in ICP usually Ar gas, to create a fine mist; the aerosol. Ultrasound nebulizers are able to improve detection limits by as

much as a factor ten as compared to pneumatic ones, but in return they are significantly more expensive (Thomas, 2001e;Gaines, 2005).

Spray chambers are commonly found in two designs: double pass and cyclonic. The double pass design is a tube fitted with a smaller, central tube, and while the aerosol is travelling the length of the latter, the larger, and heavier, droplets fall to the bottom due to gravity and are drained away. The smallest droplets are forced back through the space between the inner and outer tubes and continue to the torch. In the cyclonic design, centrifugal forces are used to separate heavy and light droplets before reaching the torch (Thomas, 2001e).

To create the plasma, a torch, a radio frequency (RF) coil, an RF source and a gas are needed. The torch usually consists of three concentric tubes inside each other, creating three “spaces”. Between the outer wall and the first inner tube flows the gas that subsequently forms the plasma. Between first and second inner tubes is a flow of auxiliary gas and in the innermost tube is the sample aerosol and a carrier, or nebulizer, gas. All three gases are usually Ar, but other gases or mixtures may be used in some cases. Around the end of the torch is the RF coil, powered by the RF source. Alternating current within the coil leads to the creation of an electromagnetic field, which is able to strip off electrons from the Ar atoms in the gas. Collisions between these electrons and other Ar atoms cause more electrons to be stripped off in a chain reaction (Thomas, 2001a). This leads to the formation of a plasma, which may reach a temperature of up to 10,000K, and into which the sample aerosol is led. In this heat, remaining liquid is evaporated from the sample mist, forming at first very small, solid particles. These solids are subsequently turned into a gas, and then atomized. Collisions with electrons arising mainly from the Ar gas lead to the formation of positively charged ions, which continue into the interface region and further into either the mass spectrometer (in ICP-MS) or the optical emission spectrophotometer (in ICP-OES) (Taylor, 2001;Thomas, 2001a;Harris, 2007).

3.4.3 Detection of Atoms

Atomic Absorption Spectroscopy

All free atoms absorb light at specific frequencies. This is exploited in AAS by placing a hollow cathode lamp with a cathode made of the analyte element just before the flame or GF. The excited atoms sputtered off from the lamp emit light at the exact same frequency as what is absorbed by the analyte atoms in the flame or GF. The difference between the intensity of light passing around the flame and passing through it therefore defines the absorption, and using a calibration curve based on a dilution row, this is related to elemental concentrations in the sample (Hanlon, 1998;Harris, 2007). The procedure is repeated for each selected element, with a change of lamps. In newer instruments, this change is done smoothly, making the analysis *de facto* multi-elemental. An overview of the experimental setup is given in Figure 15.

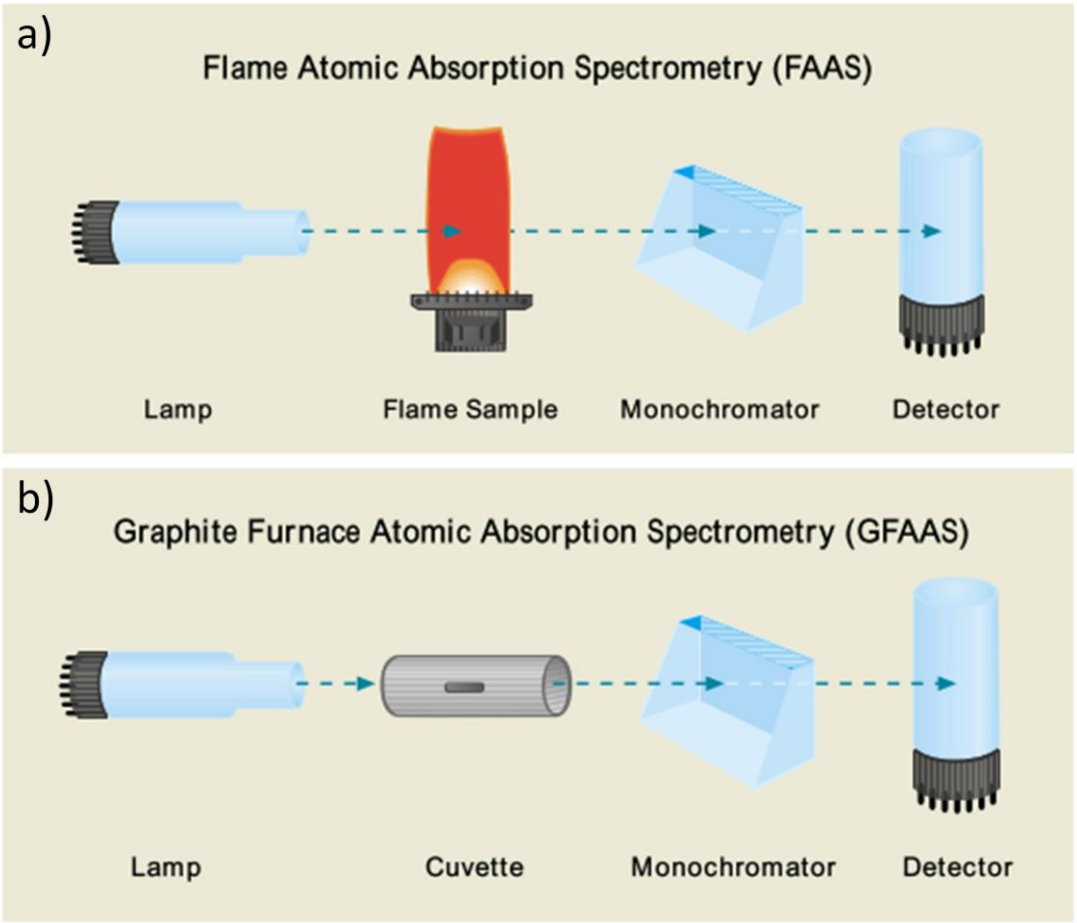

Figure 15. Overview of the AAS setup using either flame ionization (a) or graphite furnace (b). From Thermo (2013).

The Continuum Source-GF-AAS (CS-GF-AAS) is a newer method, which approaches actual multi-elemental measurements. The setup resembles that of F-AAS and GF-AAS, but the light source is a xenon lamp, which provides high light intensities in the visible and ultraviolet (UV) regions. After passing through the GF, the light is grated by a monochromator and subsequently detected by a Charge Coupled Device (CCD) array to quantify the amounts and wavelengths of absorbed light. In theory, this setup should enable multi-elemental analysis; however, further development of the detector is essential to obtain this. At present, the bandwith of the detector is <1nm, meaning that only elements with very close absorption lines may be detected simultaneously (Resano and Garcia-Ruiz, 2011).

In AAS, non-metal elements cannot be detected; meaning for instance macronutrients as N and S. Heavy metals and transition metals, *e.g.* Mn, Fe, Cu and Zn, on the other hand are atomized and measured very well. For refractory elements, *e.g.* Mo and B, the flame temperature in F-AAS is not high enough to break down compounds of these elements. In GF-AAS, the slightly higher temperature combined with the longer residence time improve detection limits by as much as a factor 1000 as compared to F-AAS in some cases. However, for refractory elements, performance is still limited (Thermo, 2013).

Optical Emission Spectroscopy

The basis for the OES technique is the excitation of an electron from an atom or an ion to a higher level of energy. During the following return to their ground state (relaxation), light is emitted at wavelengths characteristic for each element. Directly after sample ionization in the plasma, ions are excited as a result of collisions with energetic electrons derived from the Ar gas. When the excited electrons relax, the emitted light is grated and the various wavelengths are detected. The resulting spectrum is employed for calculation of total elemental concentrations in the sample by use of a calibration based on standard solutions. As one spectrum contains information regarding numerous elements, the technique is multi-elemental. An overview of an ICP-OES instrument is shown in Figure 16 (Boss and Fredeen, 1997).

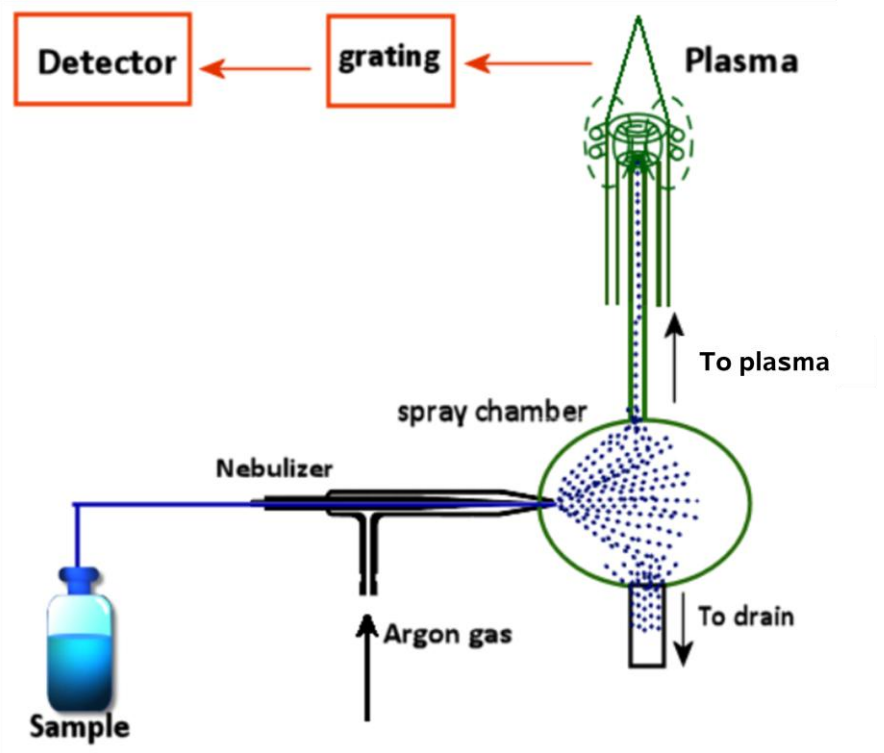

Figure 16. Overview of the ICP-OES, from sample introduction to detection. From Chemiasoft (2013).

Mass Spectrometry

In an ICP-MS instrument, the ions enter an interface region after ionization in the plasma. The purpose of this region is to align and focus the ion stream while leading it from the plasma at atmospheric pressure, to the MS-region under vacuum. The ion stream is lead through two cones, first a sampler cone, with an orifice of 0.8-1.2 mm, then a skimmer cone, with an orifice of 0.4-0.8 mm (Taylor, 2001;Thomas, 2001b) and further directed through an ion focusing system. The exact design of this may vary much between manufacturers, but the role of the system is to transport as many ions to the detector as possible while at the same time avoiding matrix components and non-analyte-based species. This is done using one or more electrodes in the shape of metallic plates, barrels or cylinders, which stop protons and neutral species (Taylor, 2001;Thomas, 2001c).

The final selection according to mass to charge ratio takes place in a quadropole consisting of four identical rods. Currents are placed on pairs of rods, direct current on one pair, an RF field on

the other, enabling only ions of a specific mass to charge ratio to travel through the center of the quadropole to reach the detector at the end, where the number of ions is counted. Changing the currents of the quadropole enables passage of other elements, rapidly after each other, making the MS a multi-elemental method. By means of a calibration based on standard solutions, elemental concentrations are calculated (Taylor, 2001;Thomas, 2001d). An overview of the ICP-MS is provided in Figure 17.

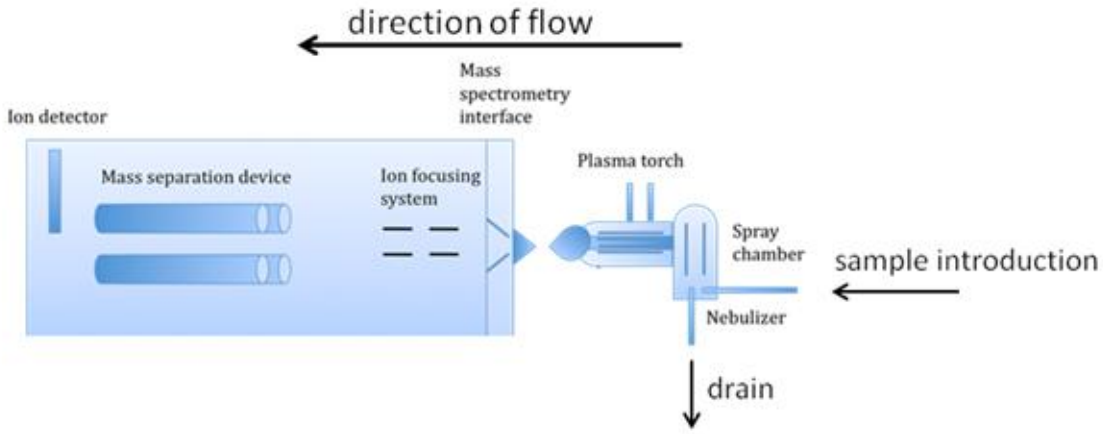

Figure 17. Overview of the ICP-MS, from sample introduction to detection. Modified from Jebb and Barron (2013).

ICP-OES and ICP-MS are able to measure both metal and non-metal elements, though not N, due to high background levels of atmospheric N, and C, which is removed already during digestion.

3.5 Fast Spectroscopic Methods

Fast spectroscopy to determine plant nutritional status is a field in rapid development. Several methods are already available and used for plant analysis directly on plants in the field. Assessment of N status of crops by tractor mounted or hand-held equipment is being used increasingly, a hand-held device for detection of Mn deficiency exists, and related equipments for assessment of *e.g.* grape maturity are also on the market, to mention a few (Force-A, 2010;Cerovic et al., 2012;NutriNostica, 2013;Yara, 2013). At the same time, intensive research is undertaken to improve existing and develop new and better techniques.

Fast, spectroscopic techniques offer rapid analysis with instant results, enabling in some cases tractor mounting with a direct link to fertilizing equipment. In most cases, the methods require no or only little sample pre-treatment and they are often non-destructive. Once the equipment has been acquired, each measurement is essentially free, as no chemicals or disposables are necessary. The measurements are in most cases indirect, which means that they do not measure nutrient concentrations directly. Instead, they measure compounds that relate to physiological effects derived from plant nutritional status, and in some cases, within certain limits, this may be related to plant nutrient concentrations. Therefore, the methods need to be thoroughly tested to ensure that they yield information specifically about status of the given nutrient. This includes considering if biotic or abiotic stresses may interfere with the results. An insight into the basics of the different spectroscopic methods is given below, in order to provide an understanding of the practical applications.

3.5.1 Ultraviolet, Visual, Near- and Mid-Infrared Spectroscopy

Absorption of light in the ultraviolet-visual (UV-Vis, 200-780 nm), Near Infrared (NIR, 780-2500nm) and Mid Infrared (MIR, 2500-50,000nm) parts of the electromagnetic spectrum reflects concentrations of specific molecules in a sample and is therefore routinely used for fast analysis, in science as well as in industry. Measuring UV-Vis absorption is based on electronic energy transitions within molecules. Electrons in molecular bonds can be excited by absorbing the energy from light of UV-Vis wavelengths, the main electron acceptors being single bonds (σ electrons), multiple bond (π electrons) and unshared electron pairs (Herman, 2000). The degree of absorption of UV-Vis light of a sample, thus, contains information about the sample concentrations of molecules containing these bonds. Reflectance of Vis light of a sample furthermore indicates the sample color directly.

The basis for NIR and MIR spectroscopy is molecular vibrations. For a molecule to vibrate and thereby yield an absorption signal when exposed to NIR or MIR light, it needs to have a dipole moment, or a degree of asymmetry. This means that symmetric molecules as H_2, Cl_2 or H_2-C-C-H_2 are not NIR or MIR active, whereas asymmetric molecules as CH3-CH2-OH and H_2O are. The frequency of molecular vibrations depends on the strength of chemical bonds and the mass of each atom involved. Incoming light with a frequency corresponding to that of the molecular vibrations is absorbed, and the remaining is either reflected or transmitted. Measuring either, thus, gives information of the molecular composition of a sample (Osborne et al., 1993;Pavia et al., 2001).

In plant analysis, UV-Vis, NIR and MIR spectroscopy can be used for fast elemental analysis if a stable correlation between a mineral nutrient and a spectroscopically active compound is present. The mineral may form part of the compound or be essential in its biosynthesis to obtain this. However, any excess of a nutrient will generally not be incorporated into spectroscopically active compounds and therefore not be detectable (Huang et al., 2009). Likewise, it could be anticipated that at very low concentrations, a linear relationship between nutrient concentrations and spectroscopic data would fade due to additional, negative side-effects. The indirect nature of spectroscopic methods when applied for plant elemental analysis causes an essential need for validation not only of the concentration range in which applying the method is reasonable, but also for the specificity of the method, as the spectra may be influenced by factors such as diseases, drought or deficiencies of other nutrients (Zillmann et al., 2006).

Where Vis-NIR is widely used in plant analysis, UV-Vis alone is less commonly applied for this purpose. One example of using Vis is, however, the L*a*b* or CIELAB system. Detecting the red/green color distribution (a*), the blue/yellow distribution (b*) and the lightness (L*) of reflected light from a fresh leaf sample in the Vis range has been found to characterize plant status, qualitatively, of several nutrients very well. For instance, N, P, Mg and Fe deficient maize plants could be separated from control samples when still in a latent stage, *i.e.* before development of visual deficiency symptoms (Graeff et al., 2001).

3.5.2 Chlorophyll Detection by Vis-NIR

Vis and NIR are by far the most commonly used wavelength ranges for measurements in plants, very often in combination. A number of handheld gauges using Vis-NIR detection for chlorophyll determination in plants are commercially available (Inc., 2011;Hansatech, 2013;Spinoff, 2013). The SPAD (Soil-Plant Analysis Development) chlorophyll meter was the first to be developed

already in the 1980's. This instrument measures transmittance of light at 650 and 940 nm through a leaf and relates the ratio of the two measurements to chlorophyll concentration (Inc., 2011). However, no fixed or strictly linear ratio between SPAD readings and chlorophyll concentrations has been found (Uddling et al., 2007), and a continuous development of alternative chlorophyll and growth parameters has taken place.

Tractor-mounted instruments for determination of N status of a crop, based on chlorophyll concentrations, are used today in practical agriculture. Two examples are the Yara N-sensor™ and the GreenSeeker®. These instruments measure and calculate the Normalized Difference Vegetation Index (NDVI) defined as

$$NDVI = \frac{(R_{NIR} - R_{red})}{(R_{NIR} + R_{red})}$$

Where R_{NIR} and R_{red} designate reflected light at specific wavelengths in the NIR and red (620-700 nm) parts of the electromagnetic spectrum, respectively. The exact wavelengths vary between instruments and may be shifted for customization to different crops, and related indexes may be calculated in comparable instruments. The idea behind the NDVI is that chlorophyll absorbance is high in the red part of the electromagnetic spectrum and low in the beginning of NIR. A ratio between the two gives an approximation of the chlorophyll concentration in the leaf. This is useful in many contexts, but as previously discussed, it is not a specific measure of the N status of plants (Zheng et al., 2009;Römheld, 2012). In addition, pigmentation varies significantly between plant genotypes, which should be taken into account. In fields where N is in fact the growth limiting factor, use of systems, hand held or tractor mounted, to distribute N fertilizer in accordance to chlorophyll concentration will optimize yields, as the use of the fertilizer is maximized. However, where other factors cause chlorophyll concentrations to decrease, the use of such systems has been shown to cause a decrease in nitrogen use efficiency with a risk of increased N leaching and no improvement of yields (Zillmann et al., 2006).

3.5.3 Vis-NIR for Nutrient Management

It has been attempted to use Vis-NIR spectroscopy to determine concentrations of most essential plant nutrients in numerous species, commonly using chemometrics to relate spectral information to nutrient concentrations. An overview of specific wavelengths and plant materials used in selected papers is provided in Table 3. The papers are chosen to present work on a broad range of essential nutrients. Only one paper uses mainly Vis data, combined with the lowest part of NIR (Menesatti et al., 2010), whereas the remaining focus exclusively on NIR or a combination of Vis-NIR. The information contained in Vis-NIR spectra from leaves reflect the concentrations of compounds, of which some may be related to the nutritional status of the plant. In the reviewed papers, no investigations have been carried out regarding which exact compounds are measured, though speculations based on spectral inspections combined with theoretical knowledge occur. This aspect will therefore only be sparsely covered in the present review.

Table 3. Overview of wavelength ranges and plant materials used in the papers reviewed for Vis-NIR calibrations to determine nutrient status.

Author	Wavelength Range (nm)	Plant Material	Nutrients
Menesatti et al. (2010)	400 - 1100	Fresh orange leaves	N, P, K, Ca, Mg, Fe, Mn, Zn
van Maarschalkerweerd et al. (2013)	1000 - 2500	Fresh barley leaves	Cu
Gonzalez-Martin et al. (2007)	1100 - 2000	Ground alfalfa	P, K, Ca, Fe, Mn, Zn
Agnew et al. (2004)	400 - 2500	Dry, ground ryegrass	N
Chen et al. (2002)	400 - 2500	Dry, ground sugarcane leaves	P
Cozzolino and Moron (2004)	400 - 2500	Dry, ground lucerne and clover	S, Fe, Mn, Zn, Cu, B
Dealdana et al. (1995)	1100 - 2500	Dry, ground grasses	N, P, K, Ca, Mg, Fe, Mn, Zn, Cu
Huang et al. (2009)	400 - 2500	Dry, ground or cut wheat and rice straw	K, Ca, Mg, Fe
Liao et al. (2012)	1100 - 2500	Dry, ground tree leaves	N, P, K, Fe, Mn, Zn, Cu
Petisco et al. (2005)	1100 - 2500	Dry, ground tree leaves	N, P, Ca
Petisco et al. (2008)	1100 - 2500	Dry, ground tree leaves	K, Mg, Fe, Zn, Cu
Ward et al. (2011)	830 - 2500	Dry, ground grasses	N, P, K
Villatoro-Pulido et al. (2012)	400 - 2500	Freeze-dried, ground rocket leaves	K, Ca, Mg, Fe, Mn, Zn, Cu

The indirect correlation between NIR spectra and nutrient concentrations means that caution must be taken during development and use of calibrations. The specificity of a calibration must be ascertained, necessitating that at least the most relevant other stresses that could interfere with the results are tested for interference with the calibration. This is rarely done, though van Maarschalkerweerd et al. (2013) demonstrated that at least for Cu, it is possible to develop a specific method based on NIR spectra.

Table 4 provides an overview for the reviewed papers of the Residual Prediction Deviations, or Ratio of Prediction to Deviation (RPD), which are standard deviations of data divided by root mean squared error of predictions (RMSEP) or root mean squared error of cross validation (RMSECV), representing the average error on predicted or cross-validated values, respectively. The RPD thus relates calibration performance to the range of measurements and is often used as an indicator of calibration performance (Ward et al., 2011;Williams, 2014). It does not, however, contain any information about the quality of the validation set, which can be highly variable, from a random subset of the same dataset as the calibration to samples collected in a different growing season. Furthermore, interpreting the quality of calibrations using RPD values is done using a variety of different schemes, as seen in *e.g.* Chen et al. (2002);Gonzalez-Martin et al. (2007);Huang et al. (2009) and Ward et al. (2011). The higher the RPD value, the better the calibration, but deciding whether it should surpass a value of 2, 3 or even 5 to be “good enough” will always depend on the intended use, why such qualitative assertions are not included in this review.

Mineral elements in plants can be detected by NIR either as part of a metallo-complex or by their influence on plant metabolism and hence the concentrations or structure of specific metabolites. Especially the composition and distribution of complexes, including the major complexes in which trace elements are contained, may vary between and within crop types as

well as in response to nutritional disorders or other biotic or abiotic stresses (Clark et al., 1987;Clark et al., 1989;Cozzolino and Moron, 2004;Villatoro-Pulido et al., 2012). This means that wavelengths of main importance to specific NIR calibrations will differ much between crops, and versatility of calibrations is, thus, often limited. Combining data from rocket leaves (*Eruca vesicaria* subsp. *sativa* and subsp. *vesicaria*) with a genetic origin in 11 widely diverse geographical regions (Villatoro-Pulido et al., 2012) resulted in NIR calibrations, which for Ca, Mg and Mn performed considerably poorer than in the other publications reviewed. Calibrations for Cu, Zn and Fe obtained RPD values in the lowest ranges of what was achieved in other publications, and only the performance of the K calibration was average compared to that of other publications (Table 4). On the other hand, combining data from up to 18 different tree species, including deciduous and evergreen species, grown in mountainous, riparian and dry areas, did not affect calibration performance negatively for most macro- and micronutrients as compared to other publications (Petisco et al. (2008), Petisco et al. (2005) and Table 4). Thus, combining various species, geographical origins and growth conditions is possible in some cases, but it is yet a factor to investigate thoroughly before employing an NIR calibration in any practical association, be it research or agriculture.

Table 4. Overview of calibration performances in the reviewed papers. Standard deviation / RMSEP, or RPD, is provided for all relevant elements. In four cases (Agnew et al., 2004;Gonzalez-Martin et al., 2007;Villatoro-Pulido et al., 2012;van Maarschalkerweerd et al., 2013), RMSEP was not available, why RPD is based on RMSECV instead. Well performing calibrations are characterized by high RPD values. The quality of validations is highly variable, why the RPD values should be interpreted with caution.

	RPD										
Author	**N**	**P**	**K**	**Ca**	**Mg**	**S**	**Fe**	**Mn**	**Zn**	**Cu**	**B**
Menesatti et al. (2010)	2.3	0.7	6.1	1.5	2.0		2.8	3.7	2.7		
van Maarschalkerweerd et al. (2013)										1.4	
Gonzalez-Martin et al. (2007)		2.4	2.3	1.5			2.1	1.4	1.7		
Agnew et al. (2004)	6.5										
Chen et al. (2002)		1.7									
Cozzolino and Moron (2004)						5.6	1.7	1.3	0.6	0.9	1.8
Dealdana et al. (1995)	3.9	1.5	1.8	2.2	1.9		1.8	1.5	1.9	1.8	
Huang et al. (2009), cut			1.7	1.8	2.1		1.3				
Huang et al. (2009), milled			2.6	2.3	2.6		1.5				
Liao et al. (2012)	2.5	1.4	1.2				1.7	1.5	1.0	1.0	
Petisco et al. (2005)	4.3	2.3		3.8							
Petisco et al. (2008)			2.4		2.2		<3		<3.1	<2.7	
Ward et al. (2011)	1.8	1.4	1.8								
Villatoro-Pulido et al. (2012)			1.9	0.8	1.1		1.2	0.9	1.3	0.9	

Several authors find that calibrations for micronutrients generally perform poorer than calibrations for macronutrients (Petisco et al., 2005;Petisco et al., 2008;Huang et al., 2009;Liao et al., 2012). This is somewhat supported by Table 4, where RPD values above 2 are almost exclusively found for macronutrient calibrations, though values below 2 also occur for macronutrients in eight out of 13 publications. The reason for the poorer performance of

micronutrient calibrations is likely to be the lower concentrations, which lead to a smaller signal to noise ratio and make spectral variation caused by differences in particle size more prone to affect calibrations (Yeh et al., 2004;Huang et al., 2009). In most investigations, NIR spectra are measured on dried, ground plant material. In addition to the homogeneity of the sample, this benefits from avoiding interference from water, which has a very dominant signal in NIR spectra. The advantage of a completely dry, homogenous sample for both macro- and micronutrient calibrations is emphasized by Huang et al. (2009), who demonstrated that RPD values of K, Ca, Mg and Fe calibrations increased significantly by measuring on dry, milled straw samples instead of cut straw (Table 4). In other words, as standard deviations were the same, RMSEP values decreased as a result of drying and milling.

When approaching sufficiency levels, the main part of the variation in a nutrient concentration is likely to be in a non-metabolic pool, such as in vacuolar storage, rather than in a pool of metabolically or structurally active nutrient, which may yield a signal in Vis-NIR (Lauer et al., 1989;Huang et al., 2009). This means that a significant part of a given nutrient in well-supplied plants is likely to stay undiscovered by the Vis-NIR measurements causing the calibration to be skewed or simply perform poorer. In a few cases (Ward et al. (2011) for P and Cozzolino and Moron (2004) for S), a vague tendency of the predictions to approach a constant value at some level above the sufficiency threshold concentration can be noticed, as exemplified in Figure 18, though this is not commented by the authors. The critical concentration threshold for P in grasses is between 0.2 and 0.3%, depending on species (Campbell, 2009); this investigation using various species of meadow grasses sampled throughout four growing seasons.

Cozzolino and Moron (2004) appear to have approximately a factor 10 error in their S reference values, which does not affect calibration performance, but obviously results cannot be compared directly to other publications.

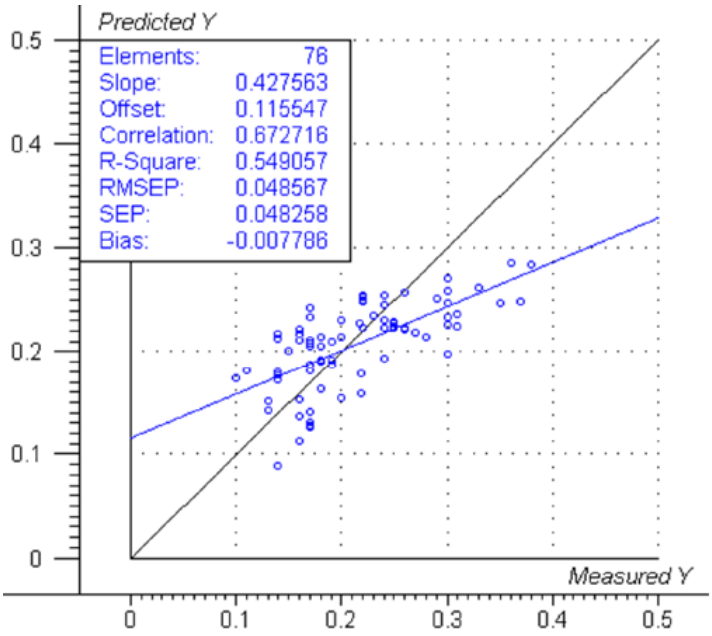

Figure 18. Predicted versus measured plot of P concentrations for a true validation set (Ward et al., 2011). Work was based on meadow grass sampled throughout four growing seasons. The unit of X and Y axes is %P in dry matter. The black line indicates Y=X, the blue line is the best linear correlation between data.

3.5.4 Nutrient Influence on Vis-NIR spectra

In section 3.5.2, several Vis-NIR based on-the-go sensors for chlorophyll detection, linked to N concentrations, are presented. Multivariate calibrations, typically Partial Least Squares (PLS), for N concentrations to a high degree also make use of the correlation between N and chlorophyll. In addition to chlorophyll, N calibrations have been shown to depend on N-H and peptide bonds of proteins, indicating a more solid correlation to N concentrations (Dealdana et al., 1995;Petisco et al., 2005). Calibrations for Mg, the central atom in chlorophyll, also rely on chlorophyll signal in Vis-NIR calibrations (Dealdana et al., 1995;Tremblay et al., 2009). However, the proportion of total plant Mg bound in chlorophyll is highly variable. For a fully Mg-supplied plant, at little as below 6% of the Mg content may be bound in chlorophyll. During Mg deficiency this proportion can increase up to 35% , and in combination with low light conditions, which increase chlorophyll concentrations, more than 50% of the total plant Mg may be bound in chlorophyll (Hawkesford et al., 2012). This variability weakens the strength of an NIR-based calibration very much. That numerous factors in addition to Mg and N deficiencies may also affect the chlorophyll concentration is clearly exemplified by Ward et al. (2011), who as the only of the reviewed investigations tested the developed models on a validation set derived from an independent growth season. This resulted in a validation error of the N calibration of 3.2, which is between three and six times more than in the other publications mentioned, and an RPD of 1.8 (Table 4), which is clearly the lowest. These figures may, however, be closer to what can be expected if using the method in practice and a comparable increase in error, meaning a decrease in RPD, may be expected for Mg calibrations if validated in a similar fashion.

Variations of P supply within deficiency to sufficiency ranges mainly affect the concentrations of major P fractions as lipids and esters, which are detectable using NIR. Also the absorption frequency for amino-acids, which are part of protein-P bonds, has been found to be of importance for P calibrations (Petisco et al., 2005;Hawkesford et al., 2012). Only at excessive P levels, the fraction of P_i increases significantly (Hawkesford et al., 2012), why a calibration set including large variations of P levels, excluding excessive levels, would be expected to yield a relatively precise NIR calibration. However, according to Table 4, P calibrations tend to perform poorer than other macronutrient calibrations. Further investigating the influence of data range on P calibration performance would be interesting, considering the possible change in effects around the sufficiency threshold (Figure 18), and because calibrations focusing exclusively on lower concentrations theoretically could perform very well.

One effect of Cu deficiency is an impaired lignification of cell walls (Broadley et al., 2012), but this has been found to occur only at severe Cu deficiency (van Maarschalkerweerd et al., 2013). During stress, also the composition of lignin may change (Gou et al., 2008), which is likely to be detectable using Vis-NIR, and it could be speculated that this is what happens during milder Cu deficiency. However, the signal from this is not sufficient to develop a quantitative calibration. An alternative approach for successfully diagnosing latent Cu deficiency in barley has been developed using supervised or unsupervised classification methods to separate NIR spectra from deficient and healthy control plants already at very early stages of deficiency. It is demonstrated that deficiencies of a number of other nutrients do not affect the diagnostic power of this method, thus, specificity is established. Using the same measurements, this paper also finds that total Cu concentrations are only poorly assessed using NIR (van Maarschalkerweerd et al., 2013).

The remaining micronutrients investigated, Fe, Mn, Zn and B, generally result in calibrations with poor prediction power. The only exception is found in Menesatti et al. (2010), where measurements are performed directly on fresh leaves and almost only Vis spectra are included, and it could, hence, be speculated that this approach is advantageous for micronutrients. However, as standard deviations of data in general are extremely low in this publication compared to other, the better performance is expected, and a comparison to other publications is not feasible.

Vis-NIR methods carry a large potential for assessment of the nutritional status of crops. Measurements are much faster than traditional laboratory analysis, even if drying and grinding has to be performed. Furthermore, the use of toxic, and expensive, chemicals is avoided. However, there is a lot of work to be done in verifying specificity of methods and assessing concentration ranges and extent of application for each single calibration. Due to the indirect nature of the Vis-NIR nutrient calibrations, this should be subject to continuous investigation during development and application of methods.

3.5.5 Fluorescence Spectroscopy and Chlorophyll Fluorescence

Fluorescence spectroscopy is based on electronic energy transitions and is thereby related to UV-Vis spectroscopy. Incoming energy, in the form of light, can excite molecules from the ground state to an excited state. However, where UV-Vis spectroscopy measures the absorption of light and thereby the excitation of molecules, fluorescence is an emission of light during relaxation. Fluorescence is a highly sensitive form of spectroscopy, being able to detect very low intensities of light. An intuitive explanation of this is that light seen on a dark background is much clearer than an increased amount of darkness, *i.e.* light absorption, on a background of light (Harris, 2007).

When light reaches a chlorophyll molecule, one out of three events will occur. The light may be absorbed and used for driving photosynthesis, it can be dissipated as heat or it is absorbed and re-emitted as fluorescence, *i.e.* chlorophyll fluorescence. Only between 2 and 10% of light absorbed by the plant result in chlorophyll fluorescence, but due to the competition between the three processes, chlorophyll fluorescence measurements contain information about the functionality of the photosynthesis (Maxwell and Johnson, 2000;Stirbet and Govindjee, 2011).

Figure 19 shows the so-called Z scheme, which provides an overview of the electron transport chain during photosynthesis. A thorough description of the scheme can be found in textbooks such as Buchanan et al. (2000), but briefly explained it shows how electrons are transported from water through the two photosystems via the cytochrome b_6f complex to finally reach the NADPH synthesis. Excitation of P680 in PSII provides the energy to transport electrons from water to plastoquinone A, Q_A, which is then reduced. The Q_A^- delivers the electron to plastoquinone B, then Q_B^-, after which the process is repeated to produce Q_B^{2-}. The Q_B^{2-} then detaches from PSII to join the plastoquinone pool (PQ) as PQH_2 and delivers the electrons to the cytochrome b_6f complex. From here, electrons are transported to PSI via plastocyanin, PC. Finally, on the acceptor side of PSI, electrons are transported to ferredoxin (Fd) and ferredoxin-NADP reductase, enabling synthesis of NADPH.

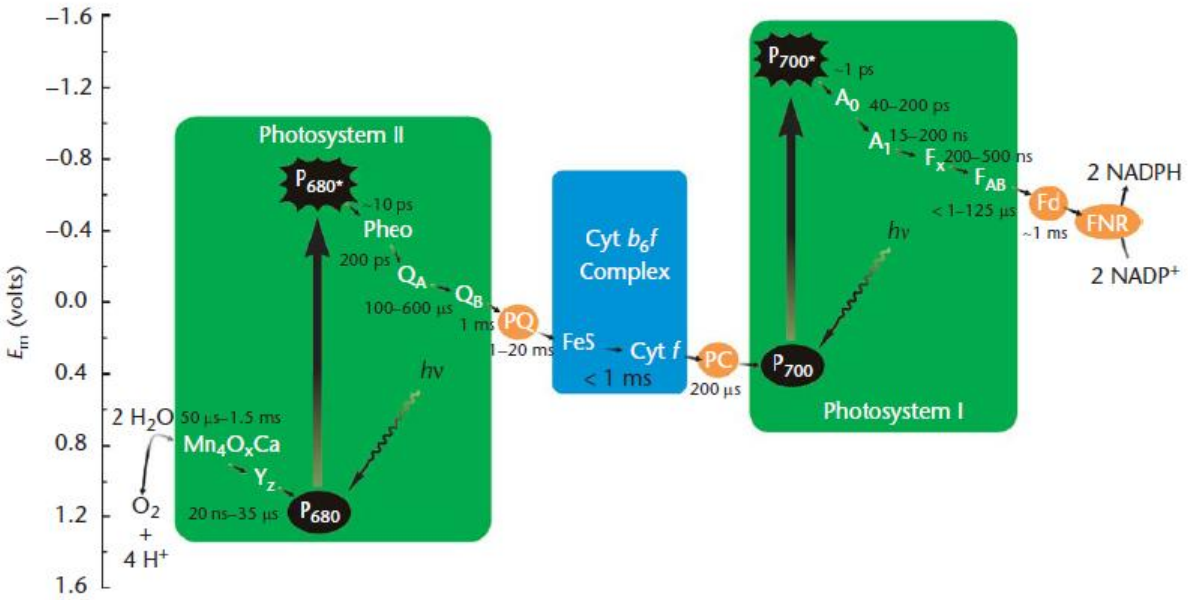

Figure 19. The Z scheme, illustrating the photosynthetic electron transport chain, which starts in PSII, then continues via the Cytochrome b6f *complex to PSI. From Govindjee et al. (2010).*

When a plant is dark adapted, all active PSII reaction centers are open, meaning that all Q_A is in the oxidized state. Exposing this plant to actinic light of weak intensity, about 0.1 µmol m^{-2} s^{-1}, yields a basic level of chlorophyll fluorescence called the O or F_0 step (Baker, 2008), which is recognized as the starting point of the transient in Figure 20. Exposing the same plant to continuous, actinic light at a high intensity causes all Q_A to be reduced within one second, and the chlorophyll fluorescence forms a curve, commonly known as the OJIP transient or the Kautsky curve. When plotted on a logarithmic time scale, the transient of a healthy plant has four steps or plateaus, known as O, J, I and P (Figure 20). P, or F_m, is the maximum fluorescence level. J, I and P steps are reached at approximately 3, 30 and 200ms, respectively (Schansker et al., 2013).

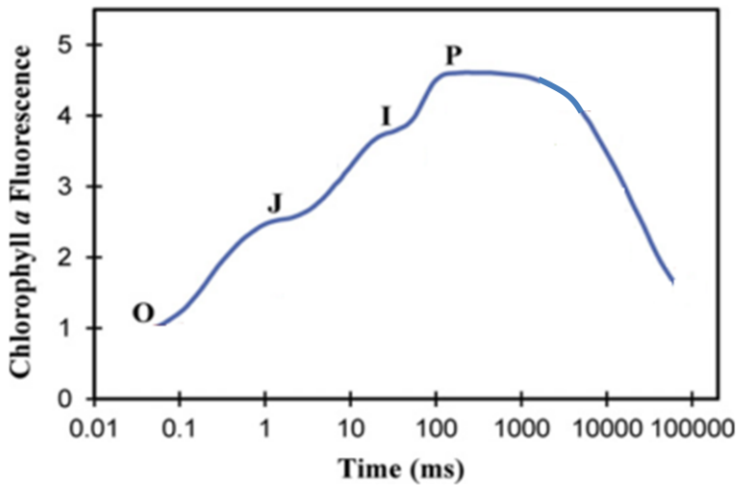

Figure 20. The OJIP transient of chlorophyll fluorescence. A dark adapted plant has been exposed to continuous, actinic light of 3200 µmol m^{-2} s^{-1}. Modified from Stirbet and Govindjee (2011).

The first phase of the OJIP transient, from O to J, is called the photochemical phase, and this is strongly affected by the intensity of the exciting light. The photochemical phase is followed by the thermal phase, which goes from J over I to the P step. The course of this phase is influenced by the temperature during measuring (Schansker et al., 2011;Stirbet and Govindjee, 2011). It has been demonstrated that the fluorescence in the photochemical phase reflects the electron acceptor side of PSII, or more exactly the first reduction of Q_A (Oukarroum et al., 2009;Yusuf et al., 2010;Stirbet and Govindjee, 2011). The J, I and P steps all seem to represent kinetic bottlenecks in the electron transport chain, and there are indications that they represent electron transport beyond PSII (Schreiber et al., 1989;Schansker et al., 2005). However, the physiological explanations of the OJIP transient, and especially the thermal phase, are much debated and further knowledge is likely to appear as investigations of the processes continue (Schansker et al., 2013).

Several stresses have been demonstrated to influence the OJIP transient, including salt, drought and heavy metal toxicity (Oukarroum et al., 2009;Yusuf et al., 2010;Adamski et al., 2011). The relation between plant nutritional status and chlorophyll fluorescence has also been investigated, and *e.g.* light scattering curves of leaves from N, P, K, Mn, Fe, S or Cu deficient sugar beets during photosynthetic induction were shown to deviate from curves of leaves from healthy control plants by visual inspection. The idea of using such measurements for fast and easy diagnosis of nutritional disorders was presented already in 1988 (Abadia et al., 1988). However, visible symptoms of the various deficiencies were pronounced at the time of measuring, why these specific results were of little practical use. A fully validated method for fast detection of Mn deficiency in barley has been developed more recently, based on determination of F_v/F_m values; F_v being the difference between F_m and F_0. In healthy control plants, this ratio is very stable around 0.83. However, rapidly after depriving plants of Mn, it declines (Hebbern et al., 2005;Husted et al., 2009). An example of chlorophyll fluorescence curves for a healthy and a Mn deficient barley plant is shown in Figure 21. The method has been validated to be specific for Mn deficiency at a time when no visual deficiency symptoms are present, if stress factors such as light and temperature can be ruled out. A small, hand-held apparatus has been developed based on this finding, and it is commonly used in Danish agriculture today, where Mn deficiency is a risk.

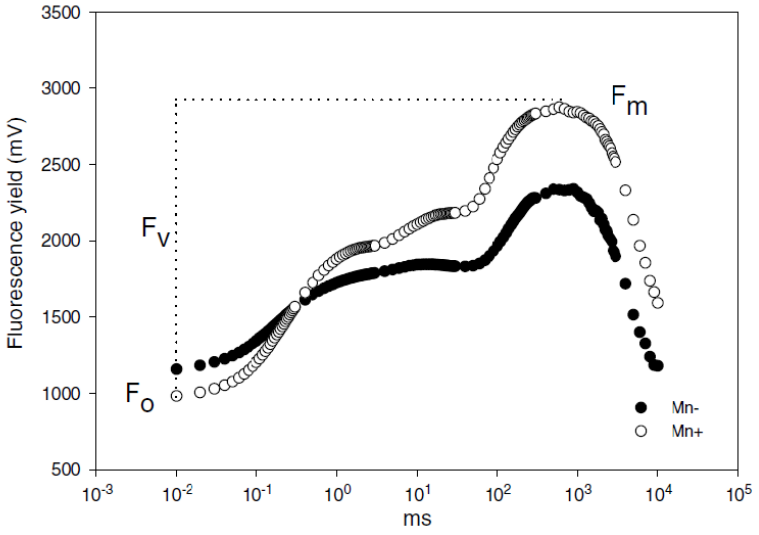

Figure 21. Chlorophyll fluorescence for Mn deficient (black circles) and Mn sufficient (open circles) barley plants at growth stage 45, i.e. *at booting. From Hebbern et al. (2005).*

In many cases, fluorescence measurements are combined with Vis-NIR. Using multivariate data analysis, this has enabled a distinction between N sufficient and N deficient potato plants in a greenhouse experiment. Also plants exposed to various levels of K and Mg deficiencies were, mostly, correctly classified according to N status (Bélanger et al., 2005). Another suggested approach for determination of N status is to use the ratio between concentrations of chlorophyll and phenolics, both of which can be determined spectroscopically (Cartelat et al., 2005). A newly developed, hand-held instrument, Dualex®, uses this methodology for assessment of N status of plants, more specifically by measuring the Nitrogen Balance Index (NBI), which is the chlorophyll concentration divided by the flavonoids concentration. Chlorophylls are determined based on transmittance measurements in the infrared and red ranges, comparable to the methods described for other N sensors (section 3.5.2). Flavonoid concentrations are described by the logarithmic ratio between infrared fluorescence at red and UV excitation light. Chlorophyll is excited by both wavelengths, whereas flavonoids only fluoresce when exposed to UV light. The NBI ensures a better correlation to N concentration than a simple measurement of chlorophyll, the level of which is affected by a number of factors, as previously discussed (Force-A, 2010;Cerovic et al., 2012).

3.5.6 X-Ray Fluorescence

X-ray fluorescence (XRF) is a spectroscopic technique for multi-elemental characterization of samples, measuring elemental concentrations directly. It exposes sample material to X-rays of appropriate energy to excite the elements in the sample, and during relaxation, X-rays of lower energy are emitted. The energy and intensity of the emitted light is characteristic for each element. For measurements in plant material, quantification of the elements is commonly done by calibrating the XRF instrument against another technique, *e.g.* ICP-OES. Generally for XRF, the heavier an element is, the easier it is to detect. Thus, heavy trace metals such as Mn, Fe, Cu, Ni and Zn are easily detectable even in very low concentrations, with limits of quantification down to a few ppm for the heaviest elements. Higher concentrations are needed for quantification of S, P, K, Mg, Ca, Cl and Na, whereas B and N are generally not detectable. Measurements are

affected by particle size and sample density, and dry plant material is therefore often ground and pelleted before measuring (Paltridge et al., 2012a;Paltridge et al., 2012b;West et al., 2012).

Using XRF can be advantageous as a lower cost alternative to ICP-OES and –MS that is also easier to operate and less sensitive to contamination, if only concentrations of heavier elements are required. Recently, XRF has been used successfully to assess Zn, Fe and Se concentrations in wheat and pearl millet grains in relation to breeding, as well as multi-elemental compositions of sunflower and alfalfa under various growing conditions (Gunes et al., 2008;Gunes et al., 2009;Paltridge et al., 2012a;Paltridge et al., 2012b). As XRF measures elemental concentrations directly, different plant species may be combined in a common calibration curve, as shown for P in cotton and maize (McLaren et al., 2012), giving the instrument a high versatility. Portable XRF instruments may have potential within plant analysis. The portable instruments are cheaper than stationary and have the theoretical advantage of working in the field. However, only theoretical, because with the common need to dry and grind samples, measuring in the field is rarely possible, leaving lower costs as the remaining advantage. Nevertheless, if a method is developed based on one relatively stable particle size, as a grain, it has been shown in rice, wheat and pearl millet that a grinding step can be omitted and good correlations for a number of elements can still be obtained (Paltridge et al., 2012a;Paltridge et al., 2012b). Where this is not possible, one of the main disadvantages of XRF with respect to plant analysis is the time consuming grinding and often pelleting of samples. In addition to this, the limited applicability for a range of essential plant elements is another disadvantage of XRF. However, in scientific or agricultural applications where focus may be on a smaller range of elements, the relatively low costs and ease of use makes XRF a good alternative to atomic spectroscopy.

3.5.7 Laser-Induced Breakdown Spectroscopy

Laser-Induced Breakdown Spectroscopy (LIBS) is a technique employing a laser beam, focused through a lens, to create a small plasma of the irradiated part of a sample surface. The plasma contains excited atomic and ionic species, which emit light as they relax to lower energy states during cooling of the plasma, which lasts only milliseconds. This light is detected and results in a spectrum with specific emission lines for the various species (Cremers and Radziemski, 2006a). A schematic overview is given in Figure 22. By use of certified reference material, the detected spectrum can be related to total concentrations of elements, much like the output from an ICP-OES.

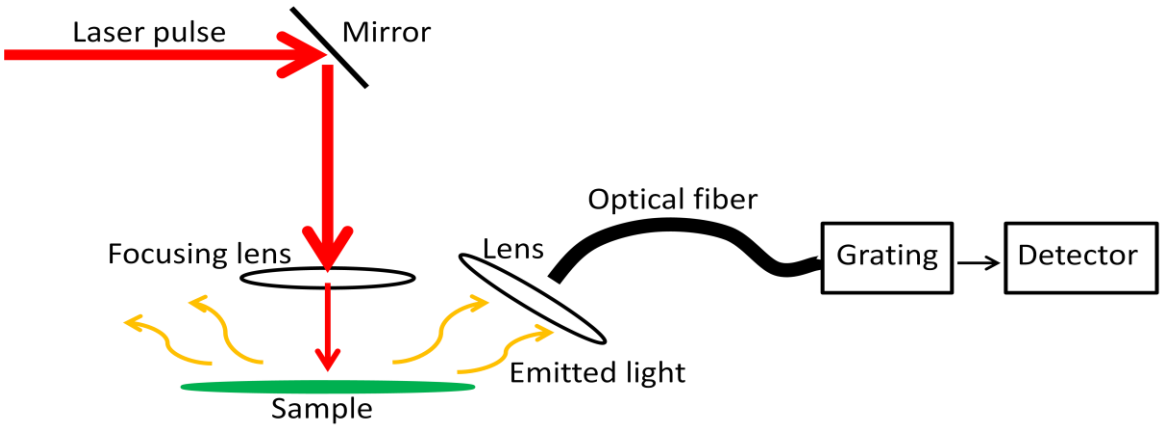

Figure 22. Schematic presentation of the LIBS technique. A laser pulse is focused using a system of mirrors and lenses, before it hits the sample. A plasma is created, and part of the emitted light is collected and led, via an optical fiber, through grating and detection.

A major advantage of the LIBS technique is the possibility of little, or even none, sample preparation. However, a few points have to be considered to obtain acceptable performance: The laser only vaporizes a very small amount of the whole sample. Significant sample inhomogeneity therefore means that measurements will not represent the whole sample. This is a particularly relevant consideration when working with plant material, where leaves include both veins and leaf blades, which may have very different elemental compositions. Also particle size distribution affects the interaction between laser and sample, why this should be optimized and standardized within a group of samples. It is therefore crucial that physical and chemical properties of the certified reference material used are comparable to those of the samples (Santos et al., 2012).

Laser Induced Breakdown Spectroscopy is still a new technique for measuring nutrients in plants, and the number of investigations is limited. However, with some success, concentrations of K, P, Mg and Ca have been measured in ground and pelleted plant material of wheat, poppy, barley and rape. The number of samples was relatively low, and K seemed to interfere with predictions of Mg and Ca in the certified reference material. This was attributed to the concentration range of K, which was considerably lower in the certified reference material than in the sample, but still, recovery values varied between 89 and 114%, which is a promising result (Pouzar et al., 2009). Effects of matrices and variations in particle size distribution and particle composition are among the most commonly mentioned problems with LIBS analyses of nutrients in plant material (Santos et al., 2012), and they are subject to thorough investigations (*e.g.* Gomes et al. (2011)). Solving these problems will obviously bring the LIBS technique forward, but it should be kept in mind that the main advantages of LIBS are still the ability to conduct fast measurements and the fact that one can measure directly on surfaces, even at a distance. Thus, any preprocessing added will limit the advantages of LIBS as compared to ICP methods. Semi-portable LIBS devices occurred already in the late 1980's and from that time, lasers and spectrographs have become more and more compact. This increases the application possibilities of LIBS markedly (Cremers and Radziemski, 2006b).

3.6 Other Techniques

New approaches to plant analysis are continuously being tested, as technological advances make them possible. The POCI array, developed by a consortium of laboratories named the Potato Oligo Chip Initiative (POCI), is an oligonucleotide potato microarray chip, which can be used to compare gene expression profiles of potato in response to stresses, for instance P deficiency. It is able to determine the expression of 42,034 potato genes simultaneously, which leads the way for multivariate data analysis yielding an overview of changes in gene expression during stresses. A major advantage of the method is the possibility to screen for a wide array of biotic and abiotic stresses at the same time. However, though the POCI has reduced time of analysis significantly, it still takes 2-3 days to obtain results, which in a practical context is relatively long (Kloosterman et al., 2008;Hammond et al., 2011), and the price may be too high for use in practical agriculture, where a large number of analyses are needed.

The term “remote sensing” is used broadly, from the visual inspection of plants over the tractor mounted sensors described in sections 3.5.2 and 3.5.3 and up to the extremes where data is collected from towers, airplanes or even satellites. The most common platforms for the latter are Vis-NIR, for generation of NDVI, or fluorescence emission. When measuring at such far distances, the investigations are mainly with the purpose of understanding ecological processes rather than precision agriculture, as the resolution naturally decreases at increasing distances. Nevertheless, Vis-NIR data detected from airborne sensors have been shown to correlate to some extent to grain yield of corn even if affected by N and P deficiencies. With time and development, the potential of air- and space-borne sensors for large scale farming may therefore be huge (Osborne et al., 2004;Malenovsky et al., 2009;Hall et al., 2011;Barton, 2012).

4 Perspectives

As the world population is continuously growing, harvest yield increases will have to grow accordingly, preferably more, to feed the world. In order to maximize yields on both fertile as well as marginal soils, optimization of nutrient management is one essential factor. Presently, soil analysis is by far the most used tool to assist plant producers in this, and a new method for soil analysis, DGT, may improve the usefulness considerably. However, monitoring of plant nutritional status throughout the growing season as well as diagnosis of acute disorders depends on accurate plant analysis. With the recent developments towards fast, easy to handle, low-cost methods, it is evident that plant analysis can play a much larger role in agricultural fertilizer management in the future than it has done up to now.

Techniques using fast spectroscopy to determine plant nutritional status still face a number of challenges related to being based on secondary correlations. Instruments are, however, already sold and used in practical agriculture, as methods have proven valuable when used appropriately. Furthermore, it demonstrates a willingness amongst plant producers to invest time and money in adapting to new ways of nutrient management, which will be a major driver towards investments in development of methods and instrumentation. The expectation of continuously increasing oil prizes, affecting as discussed the cost of mineral fertilizer itself as well as spreading of fertilizer, is another driver for development of methods to optimize the use.

Along with the development of new techniques for plant analysis, a number of perspectives arise. Using one spectroscopic technique for simultaneous determination of plant status of

several nutrients will save time and money. In addition, superior results are likely to be obtained due to the importance of interactions between different elements. As discussed, these interactions may influence plant nutritional health more than the concentration of each single nutrient.

Tractor mounting and direct linking to fertilizer equipment is already in use for N, but may be extended to other nutrients as methods are developed. For diagnosis of acute deficiencies as well as monitoring of less common deficiencies, handheld instruments have a great role to play. Furthermore, as tractor mounted instruments are costly to acquire and therefore at first will be accessible only on larger farms in high-income countries, handheld instruments may be the cheaper solution for smaller and lower income farms.

5 References

Abadia, J., Rao, I.M., and Terry, N. (1988). Light scattering in vivo as a tool for mineral nutrient deficiency diagnosis in crop plants. *Journal of plant nutrition* 11**,** 423-434.

Adamski, J.M., Peters, J.A., Danieloski, R., and Bacarin, M.A. (2011). Excess iron-induced changes in the photosynthetic characteristics of sweet potato. *Journal of plant physiology* 168**,** 2056-2062.

Agbenin, J.O., and Welp, G. (2012). Bioavailability of copper, cadmium, zinc, and lead in tropical savanna soils assessed by diffusive gradient in thin films (DGT) and ion exchange resin membranes. *Environmental Monitoring and Assessment* 184**,** 2275-2284. doi: 10.1007/s10661-011-2116-5.

Agnew, R.E., Park, R.S., Mayne, C.S., and Laidlaw, A.S. (2004). Potential of near infrared spectroscopy to predict the voluntary intake of grazed grass. *Animal Feed Science and Technology* 115**,** 169-178. doi: 10.1016/j.anifeedsci.2004.01.009.

Alves, B.J.R., Zotarelli, L., Resende, A.S., Polidoro, J.C., Urquiaga, S., and Boddey, R.M. (2000). Rapid and sensitive determination of nitrate in plant tissue using flow injection analysis. *Communications in Soil Science and Plant Analysis* 31**,** 2739-2750. doi: 10.1080/00103620009370623.

Anderson, J.W. (1996). Effect of sulfur nutrition on the redistribution of sulfur in vegetative soybean plants. *Plant physiology* 112**,** 623-631.

Anderson, M.K. (2005). *Tending the wild: Native American knowledge and the management of California's natural resources.* University of California Press.

Anjos, R.M., Mosquera, B., Sanches, N., Cambui, C.A., and Mercier, H. (2009). Caesium, potassium and ammonium distributions in different organs of tropical plants. *Environmental and Experimental Botany* 65**,** 111-118. doi: 10.1016/j.envexpbot.2008.04.001.

Anonymous. 2003. Vejledning i udtagning af jordprøver på landbrugsjord. [Accessed September 9, 2013].

Araujo, G.C.L., Lemos, S.G., and Nabais, C. (2009). Nickel sorption capacity of ground xylem of Quercus ilex trees and effects of selected ligands present in the xylem sap. *Journal of Plant Physiology* 166**,** 270-277. doi: 10.1016/j.jplph.2008.06.001.

Baker, N.R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu Rev Plant Biol* 59**,** 89-113. doi: 10.1146/annurev.arplant.59.032607.092759.

Bantan, T., Milacic, R., Mitrovic, B., and Pihlar, B. (1999). Combination of various analytical techniques for speciation of low molecular weight aluminium complexes in plant sap. *Fresenius Journal of Analytical Chemistry* 365**,** 545-552. doi: 10.1007/s002160051520.

Barakiva, A., and Lavon, R. (1968). Peroxidase Activity as an Indicator of Iron Requirement of Citrus Plants. *Israel Journal of Agricultural Research* 18**,** 145-153.

Barakiva, A., and Lavon, R. (1969). Carbonic Anhydrase Activity as an Indicator of Zinc Deficiency in Citrus Leaves. *Journal of Horticultural Science & Biotechnology* 44**,** 359-362.

Barakiva, A., Lavon, R., and Sagiv, J. (1969). Ascorbic Acid Oxidase Activity as a Measure of Copper Nutrition Requirement of Citrus Trees *Agrochimica* 14**,** 47-54.

Barton, C.V.M. (2012). Advances in remote sensing of plant stress. *Plant and Soil* 354**,** 41-44. doi: 10.1007/s11104-011-1051-0.

Baxter, I. (2009). Ionomics: studying the social network of mineral nutrients. *Current Opinion in Plant Biology* 12**,** 381-386. doi: 10.1016/j.pbi.2009.05.002.

Bélanger, M.C., Viau, A.A., Samson, G., and Chamberland, M. (2005). Determination of a Multivariate Indicator of Nitrogen Imbalance (MINI) in Potato Using Reflectance and Fluorescence Spectroscopy. *Agronomy Journal* 97**,** 1515-1523. doi: 10.2134/agronj2005.0040.

Bell, A., Bailey, J., and Smith, R. (2005). An evaluation of the Olsen test as a measure of plant-available phosphorus in grassland soils derived from basalt parent material. *Soil use and management* 21**,** 322-329.

Boss, C.B., and Fredeen, K.J. (1997). *Concepts, instrumentation and techniques in inductively coupled plasma optical emission spectrometry.* Perkin-Elmer New York.

Bp (2012). *BP Statistical Review on World Energy 2012* [Online]. BP. Available: http://www.bp.com/sectionbodycopy.do?categoryId=9037132&contentId=7069049#/Oil/Choose-your-own-data/?chartView=mapChart&regionID=none&countryID=none [Accessed 2013].

Broadley, M., Brown, P., Cakmak, I., Rengel, Z., and Zhao, F. (2012). "Function of Nutrients: Micronutrients," in *Mineral Nutrition of Higher Plants,* ed. P. Marschner. 3rd ed (London: Elsevier), 191-248.

Bromfield, S. (1987). Simple tests for the assessment of aluminium and manganese levels in acid soils. *Animal Production Science* 27**,** 399-404.

Brown, P.H., and Hu, H. (1996). Phloem mobility of boron is species dependent: evidence for phloem mobility in sorbitol-rich species. *Annals of Botany* 77**,** 497-506.

Browner, R.F., and Boorn, A.W. (1984). Sample Introduction - The Achilles Heel of Atomic Spectroscopy. *Analytical Chemistry* 56**,** 786A-798A. doi: 10.1021/ac00271a001.

Buchanan, B., Gruissem, W., and Jones, R. (2000). *Biochemistry and molecular biology of plants.* Rockville, Maryland: American Society of Plant Physiologists.

Bucksci. 2013. Available: http://www.bucksci.com/atomic-absorption-spectrophotometers/details/2/1/atomic-absorption-spectrophotometers/220-graphite-furnace-module.html [Accessed August 7, 2013].

Campbell, C.R. 2009. Reference Sufficiency Ranges for Plant Analysis in the Southern Region of the United States. *Southern Cooperative Series Bulletin* [Online].

Cartelat, A., Cerovic, Z.G., Goulas, Y., Meyer, S., Lelarge, C., Prioul, J.L., Barbottin, A., Jeuffroy, M.H., Gate, P., Agati, G., and Moya, I. (2005). Optically assessed contents of leaf polyphenolics and chlorophyll as indicators of nitrogen deficiency in wheat (Triticum aestivum L.). *Field Crops Research* 91**,** 35-49. doi: 10.1016/j.fcr.2004.05.002.

Cattani, I., Capri, E., Boccelli, R., and Del Re, A.a.M. (2009). Assessment of arsenic availability to roots in contaminated Tuscany soils by a diffusion gradient in thin films (DGT) method and uptake by Pteris vittata and Agrostis capillaris. *European Journal of Soil Science* 60**,** 539-548. doi: 10.1111/j.1365-2389.2009.01144.x.

Cerovic, Z.G., Masdoumier, G., Ben Ghozlen, N., and Latouche, G. (2012). A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. *Physiologia Plantarum* 146**,** 251-260. doi: DOI 10.1111/j.1399-3054.2012.01639.x.

Chang, C.W., Laird, D.A., Mausbach, M.J., and Hurburgh, C.R. (2001). Near-infrared reflectance spectroscopy-principal components regression analyses of soil properties. *Soil Science Society of America Journal* 65**,** 480-490.

Chapman, V.J., Kerven, G.L., Edwards, D.G., Asher, C.J., and Blamey, F.P.C. (1996). Determination of low boron concentrations in nutrient solution. *Plant and Soil* 180**,** 97-99. doi: 10.1007/bf00015415.

Chemiasoft. 2013. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). [Accessed August 7, 2013].

Chen, M., Chen, J., and Sun, F. (2008). Agricultural phosphorus flow and its environmental impacts in China. *Science of the Total Environment* 405**,** 140-152. doi: 10.1016/j.scitotenv.2008.06.031.

Chen, M., Glaz, B., Gilbert, R.A., Daroub, S.H., Barton, F.E., and Wan, Y. (2002). Near-infrared reflectance spectroscopy analysis of phosphorus in sugarcane leaves. *Agronomy Journal* 94**,** 1324-1331.

Clark, D., Cary, E., and Mayland, H. (1989). Analysis of trace elements in forages by near infrared reflectance spectroscopy. *Agronomy Journal* 81**,** 91-95.

Clark, D., Mayland, H., and Lamb, R. (1987). Mineral analysis of forages with near infrared reflectance spectroscopy. *Agronomy Journal* 79**,** 485-490.

Comte, I., Davidson, R., Lucotte, M., De Carvalho, C.J.R., Oliveira, F.D., Da Silva, B.P., and Rousseau, G.X. (2012). Physicochemical properties of soils in the Brazilian Amazon following fire-free land preparation and slash-and-burn practices. *Agriculture Ecosystems & Environment* 156**,** 108-115. doi: 10.1016/j.agee.2012.05.004.

Cozzolino, D., and Moron, A. (2004). Exploring the use of near infrared reflectance spectroscopy (NIRS) to predict trace minerals in legumes. *Animal Feed Science and Technology* 111**,** 161-173.

Cremers, D.A., and Radziemski, L.J. (2006a). "Handbook of Laser–Induced Breakdown Spectroscopy". John Wiley & Sons).

Cremers, D.A., and Radziemski, L.J. (2006b). "History and Fundamentals of LIBS," in *Laser Induced Breakdown Spectroscopy,* eds. A.W. Miziolek, V. Palleschi & I. Schechter. (USA: Cambridge University Press), 1-39.

Dalby, A. (1998). *Cato: On Farming. De Agricultura. A Modern Translation with Commentary.* Devon, UK: Prospect Books.

Dang, Y.P., Edwards, D.G., Dalal, R.C., and Tiller, K.G. (1993). Identification of an Index Tissue to Predict Zinc Status of Wheat. *Plant and Soil* 154**,** 161-167. doi: 10.1007/bf00012521.

Davison, W., and Zhang, H. (2012). Progress in understanding the use of diffusive gradients in thin films (DGT) - back to basics. *Environmental Chemistry* 9**,** 1-13. doi: 10.1071/en11084.

Dealdana, B.R.V., Criado, B.G., Ciudad, A.G., and Corona, M.E.P. (1995). Estimation of Mineral-Content in Natural Grasslands by Near-Infrared Reflectance Spectroscopy. *Communications in Soil Science and Plant Analysis* 26**,** 1383-1396. doi: 10.1080/00103629509369379.

Debnath, A., Barrow, N.J., Ghosh, D., and Malakar, H. (2010). Diagnosing P status and P requirement of tea (Camellia sinensis L.) by leaf and soil analysis. *Plant and Soil* 341**,** 309-319. doi: 10.1007/s11104-010-0645-2.

Delhaize, E., Loneragan, J.F., and Webb, J. (1982). Enzymic Diagnosis of Copper Deficiency in Subterranean Clover. 2. A Simple Field-Test. *Australian Journal of Agricultural Research* 33**,** 981-987. doi: 10.1071/ar9820981.

Du, C., and Zhou, J. (2008). Evaluation of soil fertility using infrared spectroscopy: a review. *Environmental Chemistry Letters* 7**,** 97-113. doi: 10.1007/s10311-008-0166-x.

Du, C., and Zhou, J. (2011). Application of Infrared Photoacoustic Spectroscopy in Soil Analysis. *Applied Spectroscopy Reviews* 46**,** 405-422.

Errebhi, M., Rosen, C.J., and Birong, D.E. (1998). Calibration of a petiole sap nitrate test for irrigated 'Russet Burbank' potato. *Communications in Soil Science and Plant Analysis* 29**,** 23-35. doi: 10.1080/00103629809369926.

Fageria, N.K., Baligar, V.C., and Li, Y.C. (2008). The role of nutrient efficient plants in improving crop yields in the twenty first century. *Journal of Plant Nutrition* 31**,** 1121-1157. doi: 10.1080/01904160802116068.

Feller, C.L., Thuries, L.J.M., Manlay, R.J., Robin, P., and Frossard, E. (2003). "The principles of rational agriculture" by Albrecht Daniel Thaer (1752-1828). An approach to the sustainability of cropping systems at the beginning of the 19th century. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 166**,** 687-698. doi: 10.1002/jpln.200321233.

Finlay, M.R. (1991). The rehabilitation of an agricultural chemist: Justus von Liebig and the seventh edition. *Ambix* 38**,** 155-166.

Fixen, P.E., and Grove, J.H. (1990). "Testing Soils for Phosphorus," in *Soil Testing and Plant Analysis,* ed. R.L. Westerman. (Wisconsin, USA: Soil Science Society of America), 141-180.

Flores, E.M.M., Barin, J.S., Mesko, M.F., and Knapp, G. (2007). Sample preparation techniques based on combustion reactions in closed vessels - A brief overview and recent

applications. *Spectrochimica Acta Part B-Atomic Spectroscopy* 62**,** 1051-1064. doi: DOI 10.1016/j.sab.2007.04.018.

Force-A (2010). "Dualex 4 - Flavonoid and Chlorophyll Meter". (http://www.force-a.eu/an/download2.html: Force-A, Orsay, France).

Franzen, D.W., and Cihacek, L.J. 1998. Soil Sampling as a Basis for Fertilizer Application. [Accessed September 9, 2013].

Fussell, G.E. (1971). *Crop nutrition: science and practice before Liebig.* Coronado Pr.

Gaines, P. (2005). Sample introduction for ICP-MS and ICP-OES. *Spectroscopy* 20**,** 20-23.

Gamuyao, R., Chin, J.H., Pariasca-Tanaka, J., Pesaresi, P., Catausan, S., Dalid, C., Slamet-Loedin, I., Tecson-Mendoza, E.M., Wissuwa, M., and Heuer, S. (2012). The protein kinase Pstol1 from traditional rice confers tolerance of phosphorus deficiency. *Nature* 488**,** 535-541. doi: 10.1038/nature11346.

Gassner, A., Fleckestein, J., Haneklaus, S., and Schnug, E. (2002). Spatial speciation - A new approach to assess soil analysis methods. *Communications in Soil Science and Plant Analysis* 33**,** 3347-3357. doi: 10.1081/css-120014528.

Gilbert, N. (2009). Environment: The disappearing nutrient. *Nature* 461**,** 716-718. doi: 10.1038/461716a.

Gomes, M.D., Santos, D., Nunes, L.C., De Carvalho, G.G.A., Leme, F.D., and Krug, F.J. (2011). Evaluation of grinding methods for pellets preparation aiming at the analysis of plant materials by laser induced breakdown spectrometry. *Talanta* 85**,** 1744-1750. doi: DOI 10.1016/j.talanta.2011.06.069.

Gonzalez-Martin, I., Hernandez-Hierro, J.M., and Gonzalez-Cabrera, J.M. (2007). Use of NIRS technology with a remote reflectance fibre-optic probe for predicting mineral composition (Ca, K, P, Fe, Mn, Na, Zn), protein and moisture in alfalfa. *Analytical and Bioanalytical Chemistry* 387**,** 2199-2205. doi: 10.1007/s00216-006-1039-4.

Goswami, K., Choudhury, H.K., and Saikia, J. (2012). Factors influencing farmers' adoption of slash and burn agriculture in North East India. *Forest Policy and Economics* 15**,** 146-151. doi: 10.1016/j.forpol.2011.11.005.

Gou, J.Y., Park, S., Yu, X.H., Miller, L.M., and Liu, C.J. (2008). Compositional characterization and imaging of "wall-bound" acylesters of Populus trichocarpa reveal differential accumulation of acyl molecules in normal and reactive woods. *Planta* 229**,** 15-24. doi: 10.1007/s00425-008-0799-9.

Govindjee, Kern, J.F., Messinger, J., and Whitmarsh, J. (2010). "Photosystem II," in *Encyclopedia of Life Sciences (ELS)*. (Chichester: John Wiley & Sons, Ltd).

Graeff, S., Steffens, D., and Schubert, S. (2001). Use of reflectance measurements for the early detection of N, P, Mg, and Fe deficiencies in Zea mays L. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 164**,** 445-450. doi: 10.1002/1522-2624(200108)164:4<445::aid-jpln445>3.0.co;2-1.

Gruhn, P., Goletti, F., and Yudelman, M. (2000). "Integrated nutrient management, soil fertility, and sustainable agriculture: current issues and future challenges". International Food Policy Research Institute).

Grundon, N.J., Robson, A.D., Lambert, M.J., and Snowball, K. (1997). "Nutrient Deficiency and Toxicity Symptoms," in *Plant Analysis - an Interpretation Manual,* eds. D.J. Reuter & J.B. Robinson. 2nd ed (Australia: CSIRO Publishing), 37-47.

Gunes, A., Inal, A., Pilbeam, D.J., and Kadioglu, Y.K. (2009). Effect of Sulfur on the Yield and Essential and Nonessential Element Composition of Alfalfa Determined by Polarized Energy Dispersive X-ray Fluorescence. *Communications in Soil Science and Plant Analysis* 40**,** 2264-2284. doi: 10.1080/00103620902961235.

Gunes, A., Kadioglu, Y.K., Pilbeam, D.J., Inal, A., Coban, S., and Aksu, A. (2008). Influence of silicon on sunflower cultivars under drought stress, II: Essential and nonessential element uptake determined by polarized energy dispersive x-ray fluorescence.

Communications in Soil Science and Plant Analysis 39**,** 1904-1927. doi: 10.1080/00103620802134719.

Hafner, S. (2003). Trends in maize, rice, and wheat yields for 188 nations over the past 40 years: a prevalence of linear growth. *Agriculture Ecosystems & Environment* 97**,** 275-283. doi: 10.1016/s0167-8809(03)00019-7.

Hall, F.G., Hilker, T., and Coops, N.C. (2011). PHOTOSYNSAT, photosynthesis from space: Theoretical foundations of a satellite concept and validation from tower and spaceborne data. *Remote Sensing of Environment* 115**,** 1918-1925. doi: 10.1016/j.rse.2011.03.014.

Hammond, J.P., Broadley, M.R., Bowen, H.C., Spracklen, W.P., Hayden, R.M., and White, P.J. (2011). Gene Expression Changes in Phosphorus Deficient Potato (Solanum tuberosum L.) Leaves and the Potential for Diagnostic Gene Expression Markers. *Plos One* 6. doi: 10.1371/journal.pone.0024606.

Hanlon, E.A. (1998). "Elemental Determination by Atomic Absorption Spectrophotometry," in *Handbook of Reference Methods for Plant Analysis,* ed. Y.P. Kalra. 1st ed (USA: CRC Press), 157-164.

Hansatech (2013). *CL-01 - HANSATECH* [Online]. http://www.gmrstrumenti.com/site/en/products/ecophysiology/chlorophyll-measurement/54-cl01-hansatech.html. [Accessed August 13 2013].

Hansen, O.M. 2002. Udtagning af positionsbestemte jordprøver. [Accessed September 10, 2013].

Hansen, T.H., Laursen, K.H., Persson, D.P., Pedas, P., Husted, S., and Schjoerring, J.K. (2009). Micro-scaled high-throughput digestion of plant tissue samples for multi-elemental analysis. *Plant Methods* 5. doi: 10.1186/1746-4811-5-12.

Harris, D.C. (2007). *Quantitative Chemical Analysis.* USA: WH Freeman.

Hartz, T.K. 2007. Efficient Nitrogen Management for Cool-Season Vegetables [Accessed September 25, 2013].

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J.K., Møller, I.S., and White, P. (2012). "Functions of Macronutrients," in *Mineral Nutrition of Higher Plants,* ed. P. Marschner. 3rd ed (London: Elsevier), 135-189.

Hazell, P.B.R. (2009). "Transforming Agriculture: The Green Revolution in Asia," in *Millions fed: Proven successes in agricultural development,* eds. D.J. Spielman & R. Pandya-Lorch. International Food Policy Research Inst), 25-32.

He, Y.Q., Terabayashi, S., and Namiki, T. (1998). Comparison between analytical results of plant sap analysis and the dry ashing method for tomato plants cultured hydroponically. *Journal of Plant Nutrition* 21**,** 1179-1188. doi: 10.1080/01904169809365476.

Hebbern, C.A., Pedas, P., Schjoerring, J.K., Knudsen, L., and Husted, S. (2005). Genotypic differences in manganese efficiency: field experiments with winter barley (Hordeum vulgare L.). *Plant and Soil* 272**,** 233-244. doi: 10.1007/s11104-004-5048-9.

Heenan, D.P., and Campbell, L.C. (1981). Influence of Potassium and Manganese on Growth and Uptake of Magnesium by Soybeans (Glycine max (L) Merr cv. Bragg). *Plant and Soil* 61**,** 447-456. doi: 10.1007/bf02182025.

Herman, H. (2000). "UV-visible, fluorescence and chemiluminescence spectroscopies in chemical process analysis," in *Spectroscopy in process analysis,* ed. J.M. Chalmers. 1st ed (Sheffield, England: Sheffield Academic Press).

Hettiarachchi, G.M., and Gupta, U.C. (2008). "Boron, Molybdenum, and Selenium," in *Soil sampling and methods of analysis,* eds. M.R. Carter & E.G. Gregorich. CRC Press), 121-135.

Hill, J., Robson, A.D., and Loneragan, J.F. (1979). Effect of Copper Supply on the Senescence and the Retranslocation of Nutrients of the Oldest Leaf of Wheat. *Annals of Botany* 44**,** 279-287.

Hoffmann, C., Stockfisch, N., and Koch, H.J. (2004). Influence of sulphur supply on yield and quality of sugar beet (Beta vulgaris L.) - determination of a threshold value. *European Journal of Agronomy* 21**,** 69-80. doi: 10.1016/s1161-030(03)00088-1.

Houba, V.J.G., Lexmond, T.M., Novozamsky, I., and Vanderlee, J.J. (1996). State of the art and future developments in soil analysis for bioavailability assessment. *Science of the Total Environment* 178**,** 21-28. doi: 10.1016/0048-9697(95)04793-x.

Huang, C.J., Han, L.J., Yang, Z.L., and Liu, M. (2009). Exploring the use of near infrared reflectance spectroscopy to predict minerals in straw. *Fuel* 88**,** 163-168. doi: 10.1016/j.fuel.2008.07.031.

Husted, S., Laursen, K.H., Hebbern, C.A., Schmidt, S.B., Pedas, P., Haldrup, A., and Jensen, P.E. (2009). Manganese deficiency leads to genotype-specific changes in fluorescence induction kinetics and state transitions. *Plant Physiology* 150**,** 825-833. doi: 10.1104/pp.108.134601.

Husted, S., Persson, D.P., Laursen, K.H., Hansen, T.H., Pedas, P., Schiller, M., Hegelund, J.N., and Schjoerring, J.K. (2011). Review: The role of atomic spectrometry in plant science. *Journal of Analytical Atomic Spectrometry* 26**,** 52-79. doi: 10.1039/c0ja00058b.

Inc., S.T. (2011). "SPAD 502 Plus Chlorophyll Meter, Product Manual". (http://www.specmeters.com/assets/1/22/2900P_SPAD_502.pdf: Spectrum Technologies Inc.).

Ingestad, T. (1987). New Concepts on Soil Fertility and Plant Nutrition as Illustrated by Research on Forest Trees and Stands. *Geoderma* 40**,** 237-252. doi: 10.1016/0016-7061(87)90035-8.

Jebb, M., and Barron, A.R. 2013. ICP-MS for Trace Metal Analysis. Available: http://cnx.org/content/m34666/latest/ [Accessed August 7, 2013].

Jones Jr, J.B. (1998). Soil test methods: past, present, and future use of soil extractants. *Communications in Soil Science & Plant Analysis* 29**,** 1543-1552.

Jorhem, L., Engman, J., Lindestrom, L., and Schroder, T. (2000). Uptake of lead by vegetables grown in contaminated soil. *Communications in Soil Science and Plant Analysis* 31**,** 2403-2411. doi: 10.1080/00103620009370594.

Jungk, A. (2009). Carl Sprengel-The founder of agricultural chemistry A re-appraisal commemorating the 150th anniversary of his death. *Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 172**,** 633-636. doi: 10.1002/jpln.200900065.

Khush, G.S. (2001). Green revolution: the way forward. *Nature Reviews Genetics* 2**,** 815-822. doi: 10.1038/35093585.

Kirkby, E. (2012). "Introduction, Definition and Classification of Nutrients," in *Mineral Nutrition of Higher Plants,* ed. P. Marschner. 3rd ed (London: Elsevier), 3-5.

Kloosterman, B., De Koeyer, D., Griffiths, R., Flinn, B., Steuernagel, B., Scholz, U., Sonnewald, S., Sonnewald, U., Bryan, G.J., Prat, S., Banfalvi, Z., Hammond, J.P., Geigenberger, P., Nielsen, K.L., Visser, R.G.F., and Bachem, C.W.B. (2008). Genes driving potato tuber initiation and growth: identification based on transcriptional changes using the POCI array. *Functional & Integrative Genomics* 8**,** 329-340. doi: 10.1007/s10142-008-0083-x.

Kongshaug, G. (Year). "Energy consumption and greenhouse gas emissions in fertilizer production", in: *International Fertiliser Society Meeting*), 1-19.

Kurvits, A., and Kirkby, E.A. (1980). The Uptake of Nutrients by Sunflower Plants(Helianthus-Annuus) Growing in a Continuous Flowing Culture System, Supplied with Nitrate or Ammonium as nitrogen-Source. *Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* 143**,** 140-149. doi: 10.1002/jpln.19801430203.

Lauer, M.J., Blevins, D.G., and Sierzputowska-Gracz, H. (1989). 31P-nuclear magnetic resonance determination of phosphate compartmentation in leaves of reproductive soybeans (Glycine max L.) as affected by phosphate nutrition. *Plant Physiology* 89**,** 1331-1336.

Lee, S., Jeon, U.S., Lee, S.J., Kim, Y.K., Persson, D.P., Husted, S., Schjorring, J.K., Kakei, Y., Masuda, H., Nishizawa, N.K., and An, G. (2009). Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proceedings of the National Academy of Sciences of the United States of America* 106**,** 22014-22019. doi: 10.1073/pnas.0910950106.

Lewis, D.C., Grant, I.L., and Maier, N.A. (1993). Factors Affecting the Interpretation and Adoption of Plant Analysis Services. *Australian Journal of Experimental Agriculture* 33**,** 1053-1066. doi: 10.1071/ea9931053.

Li, L., Li, S.M., Sun, J.H., Zhou, L.L., Bao, X.G., Zhang, H.G., and Zhang, F.S. (2007). Diversity enhances agricultural productivity via rhizosphere phosphorus facilitation on phosphorus-deficient soils. *Proceedings of the National Academy of Sciences of the United States of America* 104**,** 11192-11196. doi: 10.1073/pnas.0704591104.

Liao, H., Wu, J., Chen, W., Guo, W., and Shi, C. (2012). Rapid Diagnosis of Nutrient Elements in Fingered Citron Leaf Using Near Infrared Reflectance Spectroscopy. *Journal of Plant Nutrition* 35**,** 1725-1734. doi: 10.1080/01904167.2012.698352.

Liao, M.T., Hedley, M.J., Woolley, D.J., Brooks, R.R., and Nichols, M.A. (2000). Copper uptake and translocation in chicory (Cichorium intybus L. cv. Grasslands Puna) and tomato (Lycopersicon esculentum Mill. cv. Rondy) plants grown in NFT system. I. Copper uptake and distribution in plants. *Plant and Soil* 221**,** 135-142. doi: 10.1023/a:1004731415931.

Linderholm, K., Tillman, A.-M., and Mattsson, J.E. (2012). Life cycle assessment of phosphorus alternatives for Swedish agriculture. *Resources Conservation and Recycling* 66**,** 27-39. doi: 10.1016/j.resconrec.2012.04.006.

Loneragan, J.F., and Snowball, K. (1969). Calcium Requirements of Plants. *Australian Journal of Agricultural Research* 20**,** 465-478. doi: 10.1071/ar9690465.

Loneragan, J.F., Snowball, K., and Simmons, W.J. (1968). Response of Plants to Calcium Concentration in Solution Culture. *Australian Journal of Agricultural Research* 19**,** 845-857. doi: 10.1071/ar9680845.

Malenovsky, Z., Mishra, K.B., Zemek, F., Rascher, U., and Nedbal, L. (2009). Scientific and technical challenges in remote sensing of plant canopy reflectance and fluorescence. *J Exp Bot* 60**,** 2987-3004. doi: 10.1093/jxb/erp156.

Manlay, R.J., Feller, C., and Swift, M.J. (2007). Historical evolution of soil organic matter concepts and their relationships with the fertility and sustainability of cropping systems. *Agriculture Ecosystems & Environment* 119**,** 217-233. doi: 10.1016/j.agee.2006.07.011.

Mason, S., Mcneill, A., Mclaughlin, M.J., and Zhang, H. (2010). Prediction of wheat response to an application of phosphorus under field conditions using diffusive gradients in thin-films (DGT) and extraction methods. *Plant and Soil* 337**,** 243-258. doi: 10.1007/s11104-010-0521-0.

Maxwell, K., and Johnson, G. (2000). Chlorophyll fluorescence--a practical guide. *Journal of experimental botany* 51**,** 659-668.

Maynard, D.G., Kalra, Y.P., and Crumbaugh, J.A. (2008). "Nitrate and Exchangeable Ammonium Nitrogen," in *Soil sampling and methods of analysis,* eds. M.R. Carter & E.G. Gregorich. CRC Press), 97-105.

Mclaren, T.I., Guppy, C.N., and Tighe, M.K. (2012). A Rapid and Nondestructive Plant Nutrient Analysis using Portable X-Ray Fluorescence. *Soil Science Society of America Journal* 76**,** 1446-1453. doi: 10.2136/sssaj2011.0355.

Mclaughlin, N.B., Hiba, A., Wall, G.J., and King, D.J. (2000). Comparison of energy inputs for inorganic fertilizer and manure based corn production. *Canadian Agricultural Engineering* 42**,** 9-17.

Menesatti, P., Antonucci, F., Pallottino, F., Roccuzzo, G., Allegra, M., Stagno, F., and Intrigliolo, F. (2010). Estimation of plant nutritional status by Vis–NIR spectrophotometric analysis on orange leaves [Citrus sinensis (L) Osbeck cv Tarocco]. *Biosystems Engineering* 105**,** 448-454. doi: 10.1016/j.biosystemseng.2010.01.003.

Menzies, N.W., Donn, M.J., and Kopittke, P.M. (2007). Evaluation of extractants for estimation of the phytoavailable trace metals in soils. *Environmental Pollution* 145**,** 121-130.

Mermet, J.-M., and Poussel, E. (1995). ICP emission spectrometers: 1995 analytical figures of merit. *Applied Spectroscopy* 49**,** 12A-18A.

Millipore, E. (2013). *Test Strips, MQuant™* [Online]. http://www.emdmillipore.com/chemicals/test-strips/c_29.b.s1LRy4AAAEWl.IfVhTl?PageNumber=p0): EMD Millipore. [Accessed August 7 2013].

Mundus, S., Lombi, E., Holm, P.E., Zhang, H., and Husted, S. (2012). Assessing the plant availability of manganese in soils using Diffusive Gradients in Thin films (DGT). *Geoderma* 183**,** 92-99. doi: 10.1016/j.geoderma.2012.03.014.

Mundus, S., Tandy, S., Cheng, H., Lombi, E., Husted, S., Holm, P.E., and Zhang, H. (2011). Applicability of Diffusive Gradients in Thin Films for Measuring Mn in Soils and Freshwater Sediments. *Analytical Chemistry* 83**,** 8984-8991. doi: 10.1021/ac201780f.

Nabi, G., Rafique, E., and Salim, M. (2006). Boron nutrition of four sweet pepper cultivars grown in boron-deficient soil. *Journal of Plant Nutrition* 29**,** 717-725. doi: 10.1080/01904160600564543.

Nduwamungu, C., Ziadi, N., Parent, L.E., Tremblay, G.F., and Thuries, L. (2009). Opportunities for, and limitations of, near infrared reflectance spectroscopy applications in soil analysis: A review. *Canadian Journal of Soil Science* 89**,** 531-541.

Nelson, G.C. (2010). *Food security, farming, and climate change to 2050: Scenarios, results, policy options.* International Food Policy Research Institute.

Nowack, B., Koehler, S., and Schulin, R. (2004). Use of diffusive gradients in thin films (DGT) in undisturbed field soils. *Environmental Science & Technology* 38**,** 1133-1138. doi: 10.1021/es034867j.

Nutrinostica (2013). *NN-easy55* [Online]. http://nutrinostica.dk/Products2.aspx?pageId=8. [Accessed 130110 2013].

Osborne, B.G., Fearn, T., and Hindle, P.H. (1993). *Practical NIR spectroscopy with applications in food and beverage analysis.* New York: Longman scientific and technical.

Osborne, S.L., Schepers, J.S., and Schlemmer, M.R. (2004). Detecting nitrogen and phosphorus stress in corn using multi-spectral imagery. *Communications in Soil Science and Plant Analysis* 35**,** 505-516. doi: 10.1081/css-120029728.

Ostatek-Boczynski, Z.A., and Lee-Steere, P. (2012). Evaluation of Mehlich 3 as a Universal Nutrient Extractant for Australian Sugarcane Soils. *Communications in Soil Science and Plant Analysis* 43**,** 623-630. doi: 10.1080/00103624.2012.643845.

Oukarroum, A., Schansker, G., and Strasser, R.J. (2009). Drought stress effects on photosystem I content and photosystem II thermotolerance analyzed using Chl a fluorescence kinetics in barley varieties differing in their drought tolerance. *Physiologia plantarum* 137**,** 188-199.

Pagani, A., and Echeverria, H.E. (2011). Performance of Sulfur Diagnostic Methods for Corn. *Agronomy Journal* 103**,** 413-421. doi: 10.2134/agronj2010.0265.

Page, V., and Feller, U. (2005). Selective transport of zinc, manganese, nickel, cobalt and cadmium in the root system and transfer to the leaves in young wheat plants. *Annals of Botany* 96**,** 425-434. doi: 10.1093/aob/mci189.

Paltridge, N.G., Milham, P.J., Ortiz-Monasterio, J.I., Velu, G., Yasmin, Z., Palmer, L.J., Guild, G.E., and Stangoulis, J.C.R. (2012a). Energy-dispersive X-ray fluorescence spectrometry as a tool for zinc, iron and selenium analysis in whole grain wheat. *Plant and Soil* 361**,** 261-269. doi: 10.1007/s11104-012-1423-0.

Paltridge, N.G., Palmer, L.J., Milham, P.J., Guild, G.E., and Stangoulis, J.C.R. (2012b). Energy-dispersive X-ray fluorescence analysis of zinc and iron concentration in rice and pearl millet grain. *Plant and Soil* 361**,** 251-260. doi: 10.1007/s11104-011-1104-4.

Parent, L.E., and Dafir, M. (1992). A Theoretical Concept of Compositional Nutrient Diagnosis. *Journal of the American Society for Horticultural Science* 117**,** 239-242.

Parent, S.-É., Parent, L.E., Egozcue, J.J., Rozane, D.E., Hernandes, A., Lapointe, L., Gentile, V.H., Naess, K., Marchand, S., Lafond, J., Mattos Jr, D., Barlow, P., and Natale, W. (2013). The plant ionome revisited by the nutrient balance concept. *Frontiers in Plant Science* 4. doi: 10.3389/fpls.2013.00039.

Parks, S.E., Irving, D.E., and Milham, P.J. (2012). A critical evaluation of on-farm rapid tests for measuring nitrate in leafy vegetables. *Scientia Horticulturae* 134**,** 1-6. doi: 10.1016/j.scienta.2011.10.015.

Parry, M.a.J., and Hawkesford, M.J. (2010). Food security: increasing yield and improving resource use efficiency. *Proceedings of the Nutrition Society* 69**,** 592-600. doi: 10.1017/s0029665110003836.

Pavia, D.L., Lampman, G.M., and Kriz, G.S. (2001). *Introduction to spectroscopy.* Harcourt College Publishers.

Pedersen, J.B. (2011). "Oversigt over Landsforsøgene 2011". (Aarhus, Denmark: Videncentret for Landbrug).

Pedersen, J.B. (2012). "Oversigt over Landsforsøgene 2012". (Aarhus, Denmark: Videncentret for Landbrug).

Pennock, D., Yates, T., and Braidek, J. (2008). "Soil Sampling Designs," in *Soil sampling and methods of analysis,* eds. M.R. Carter & E.G. Gregorich. CRC Press), 25-38.

Perkinelmer. 2013. HGA 900 Graphite Furnace. [Accessed August 7, 2013].

Petisco, C., Garcia-Criado, B., De Aldana, B.R.V., Garcia-Ciudad, A., and Mediavilla, S. (2008). Ash and mineral contents in leaves of woody species: Analysis by near-infrared reflectance spectroscopy. *Communications in Soil Science and Plant Analysis* 39**,** 905-925. doi: 10.1080/00103620701881253.

Petisco, C., Garcia-Criado, B., De Aldana, B.R.V., Zabalgogeazcoa, I., Mediavilla, S., and Garcia-Ciudad, A. (2005). Use of near-infrared reflectance spectroscopy in predicting nitrogen, phosphorus and calcium contents in heterogeneous woody plant species. *Analytical and Bioanalytical Chemistry* 382**,** 458-465. doi: 10.1007/s00216-004-3046-7.

Pilon-Smits, E.a.H., Quinn, C.F., Tapken, W., Malagoli, M., and Schiavon, M. (2009). Physiological functions of beneficial elements. *Current Opinion in Plant Biology* 12**,** 267-274. doi: 10.1016/j.pbi.2009.04.009.

Piper, C.S. (1944). *Soil and plant analysis.* New York.

Pirie, A., Singh, B., and Islam, K. (2005). Ultra-violet, visible, near-infrared, and mid-infrared diffuse reflectance spectroscopic techniques to predict several soil properties. *Australian Journal of Soil Research* 43**,** 713-721. doi: 10.1071/sr04182.

Plantedirektoratet. 1994. Fælles arbejdsmetoder for jordbundsanalyser. Available: https://www.landbrugsinfo.dk/Planteavl/Jordbund/Jordbundsproever/Sider/JBPA_laboratorie_metoder.pdf?List={408edb42-9341-4b9a-b68f-0d32b7dabe64}&download=true [Accessed September 9, 2013].

Pouzar, M., Černohorský, T., Průšová, M., Prokopčáková, P., and Krejčová, A. (2009). LIBS analysis of crop plants. *Journal of Analytical Atomic Spectrometry* 24**,** 953-957. doi: 10.1039/b903593a.

Raghupathi, H.B., Reddy, Y.T.N., Rein, M.K., and Bhargava, B.S. (2004). Diagnosis of nutrient imbalance in mango by DRIS and PCA approaches. *Journal of Plant Nutrition* 27**,** 1131-1148. doi: 10.1081/pln-120038540.

Raj, G.B., and Rao, A.P. (2006). Identification of yield-limiting nutrients in mango through DRIS indices. *Communications in Soil Science and Plant Analysis* 37**,** 1761-1774. doi: 10.1080/00103620600710587.

Rao, N.R., and Ownby, J.D. (1993). Development of an ELISA for Estimation of the Copper Nutritional-Status of Wheat and Cotton. *Plant and Soil* 155**,** 453-456. doi: 10.1007/bf00025081.

Rayment, G.E. (1993). Soil Analysis - A Review. *Australian Journal of Experimental Agriculture* 33**,** 1015-1028. doi: 10.1071/ea9931015.

Rayment, G.E., and Lyons, D.J. (2012). New, Comprehensive Soil Chemical Methods Book for Australasia. *Communications in Soil Science and Plant Analysis* 43**,** 412-418. doi: 10.1080/00103624.2012.641802.

Resano, M., and Garcia-Ruiz, E. (2011). High-resolution continuum source graphite furnace atomic absorption spectrometry: Is it as good as it sounds? A critical review. *Analytical and Bioanalytical Chemistry* 399**,** 323-330. doi: 10.1007/s00216-010-4105-x.

Resano, M., Vanhaecke, F., and De Loos-Vollebregt, M.T.C. (2008). Electrothermal vaporization for sample introduction in atomic absorption, atomic emission and plasma mass spectrometry-a critical review with focus on solid sampling and slurry analysis. *Journal of Analytical Atomic Spectrometry* 23**,** 1450-1475. doi: 10.1039/b807756h.

Reussi, N., Echeverria, H., and Rozas, H.S. (2011). Diagnosing Sulfur Deficiency in Spring Red Wheat: Plant Analysis. *Journal of Plant Nutrition* 34**,** 573-589. doi: 10.1080/01904167.2011.538118.

Reuter, D.J., Robinson, J.B., and Dutkiewicz, C. (1997). *Plant analysis: an interpretation manual.* CSIRO Publishing.

Reuter, D.J., Robson, A.D., Loneragan, J.F., and Tranthimfryer, D.J. (1981). Copper Nutrition of Subterranean Clover (Trifolium-Subterraneum L cv Seaton-Park). 2. Effects of Copper Supply on Distribution of Copper and the Diagnosis of Copper Deficiency by Plant Analysis. *Australian Journal of Agricultural Research* 32**,** 267-282. doi: 10.1071/ar9810267.

Riesen, O., and Feller, U. (2005). Redistribution of nickel, cobalt, manganese, zinc, and cadmium via the phloem in young and maturing wheat. *Journal of Plant Nutrition* 28**,** 421-430. doi: 10.1081/pln-20049153.

Rodrigues, L.F., De Mattos, J.C.P., Dressler, V.L., Pozebon, D., and Flores, E.M.D. (2007). Determination of cadmium, copper and lead in alumina based catalysts by direct solid sampling graphite furnace atomic absorption spectrometry. *Spectrochimica Acta Part B-Atomic Spectroscopy* 62**,** 933-938. doi: DOI 10.1016/j.sab.2007.04.011.

Romheld, V., and Kirkby, E.A. (2010). Research on potassium in agriculture: needs and prospects. *Plant and Soil* 335**,** 155-180. doi: 10.1007/s11104-010-0520-1.

Rossel, R.a.V., Walvoort, D.J.J., Mcbratney, A.B., Janik, L.J., and Skjemstad, J.O. (2006). Visible, near infrared, mid infrared or combined diffuse reflectance spectroscopy for simultaneous assessment of various soil properties. *Geoderma* 131**,** 59-75. doi: 10.1016/j.geoderma.2005.03.007.

Römheld, V. (2012). "Diagnosis of Deficiency and Toxicity of Nutrients," in *Mineral Nutrition of Higher Plants,* ed. P. Marschner. 3rd ed (London: Elsevier), 299-312.

Salt, D.E., Baxter, I., and Lahner, B. (2008). "Ionomics and the study of the plant ionome," in *Annual Review of Plant Biology*.), 709-733.

Santos, D., Nunes, L.C., De Carvalho, G.G.A., Gomes, M.D., De Souza, P.F., Leme, F.D., Dos Santos, L.G.C., and Krug, F.J. (2012). Laser-induced breakdown spectroscopy for analysis of plant materials: A review. *Spectrochimica Acta Part B-Atomic Spectroscopy* 71-72**,** 3-13. doi: DOI 10.1016/j.sab.2012.05.005.

Schansker, G., Tóth, S.Z., Holzwarth, A.R., and Garab, G. (2013). Chlorophyll a fluorescence: beyond the limits of the QA model. *Photosynthesis research***,** 1-16.

Schansker, G., Toth, S.Z., Kovacs, L., Holzwarth, A.R., and Garab, G. (2011). Evidence for a fluorescence yield change driven by a light-induced conformational change within photosystem II during the fast chlorophyll a fluorescence rise. *Biochimica Et Biophysica Acta-Bioenergetics* 1807**,** 1032-1043. doi: 10.1016/j.bbabio.2011.05.022.

Schansker, G., Tóth, S.Z., and Strasser, R.J. (2005). Methylviologen and dibromothymoquinone treatments of pea leaves reveal the role of photosystem I in the Chl *a* fluorescence rise OJIP. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1706**,** 250-261.

Schmidt, S.B., Pedas, P., Laursen, K.H., Schjoerring, J.K., and Husted, S. (2013). Latent Manganese Deficiency in Barley can be Diagnosed and Remediated on the Basis of Chlorophyll *a* Fluorescence Measurements. *Plant and Soil* 372**,** 417-429. doi: 10.1007/s11104-013-1702-4

Schnug, E., Fleckenstein, J., and Haneklaus, S. (1996). Coca Cola(R) is it! The ubiquitous extractant for micronutrients in soil. *Communications in Soil Science and Plant Analysis* 27**,** 1721-1730. doi: 10.1080/00103629609369665.

Schreiber, U., Neubauer, C., and Klughammer, C. (1989). Devices and methods for room-temperature fluorescence analysis. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences* 323**,** 241-251.

Singh, D., Singh, K., Hundal, H.S., and Sekhon, K.S. (2012). Diagnosis and Recommendation Integrated System (DRIS) for Evaluating Nutrient Status of Cotton (Gossipium Hirsutum). *Journal of Plant Nutrition* 35**,** 192-202. doi: 10.1080/01904167.2012.636122.

Singh, U.M., Sareen, P., Sengar, R.S., and Kumar, A. (2013). Plant ionomics: a newer approach to study mineral transport and its regulation. *Acta Physiologiae Plantarum* 35**,** 2641-2653. doi: 10.1007/s11738-013-1316-8.

Skarpa, P., Kunzova, E., and Zukalova, H. (2013). Foliar fertilization with molybdenum in sunflower (Helianthus annuus L.). *Plant Soil and Environment* 59**,** 156-161.

Smil, V. (1991). Population-growth and Nitrogen - An Exploration of a Critical Existential Link. *Population and Development Review* 17**,** 569-601. doi: 10.2307/1973598.

Smil, V. (2002). Nitrogen and food production: Proteins for human diets. *Ambio* 31**,** 126-131. doi: 10.1639/0044-7447(2002)031[0126:nafppf]2.0.co;2.

Smil, V. (2004). *Enriching the earth: Fritz Haber, Carl Bosch, and the transformation of world food production.* MIT press.

Smith, P.F. (1962). Mineral analysis of plant tissues. *Annual Review of Plant Physiology* 13**,** 81-108.

Sorensen, L.K., and Dalsgaard, S. (2005). Determination of clay and other soil properties by near infrared spectroscopy. *Soil Science Society of America Journal* 69**,** 159-167.

Spinoff, N. (2013). *Chlorophyll Meters Aid Plant Nutrient Management* [Online]. http://spinoff.nasa.gov/Spinoff2009/er_10.html. [Accessed August 13 2013].

Srivastava, A.K., and Singh, S. (2008). DRIS norms and their field validation in Nagpur mandarin. *Journal of Plant Nutrition* 31**,** 1091-1107. doi: 10.1080/01904160802115359.

Stirbet, A., and Govindjee (2011). On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. *Journal of Photochemistry and Photobiology B-Biology* 104**,** 236-257. doi: 10.1016/j.jphotobiol.2010.12.010.

Sumner, M., and Beaufils, E. (1975). Diagnosis of the NPK Requirements of Sugarcane Plant Age and Season Using Beaufils' System (DRIS)-Preliminary Observations. *Proceedings of The South African Sugar Technologists' Association-JunelJuly***,** 137-141.

Sun, H.W., Li, L.X., Qiao, F.X., and Liang, S.X. (2008). Availability of lead and cadmium in farmland soil and its distribution in individual plants of dry-seeded rice. *Communications in Soil Science and Plant Analysis* 39**,** 450-460. doi: 10.1080/00103620701826589.

Tandy, S., Mundus, S., Yngvesson, J., De Bang, T.C., Lombi, E., Schjoerring, J.K., and Husted, S. (2011). The use of DGT for prediction of plant available copper, zinc and phosphorus in agricultural soils. *Plant and Soil* 346**,** 167-180. doi: 10.1007/s11104-011-0806-y.

Tandy, S., Mundus, S., Zhang, H., Lombi, E., Frydenvang, J., Holm, P.E., and Husted, S. (2012). A new method for determination of potassium in soils using diffusive gradients in thin films (DGT). *Environmental Chemistry* 9**,** 14-23. doi: 10.1071/en11070.

Taylor, H.E. (2001). *Inductively coupled plasma-mass spectrometry: practices and techniques.* Academic Press.

Thermo. 2013. Available: http://www.thermo.com/eThermo/CMA/PDFs/Articles/articlesFile_18407.pdf [Accessed August 7, 2013].

Thomas, R. (2001a). A beginner's guide to ICP-MS - Part III: The plasma source. *Spectroscopy* 16**,** 26-30.

Thomas, R. (2001b). A beginner's guide to ICP-MS - Part IV: The interface region. *Spectroscopy* 16**,** 26-34.

Thomas, R. (2001c). A beginner's guide to ICP-MS - Part V: The ion focusing system. *Spectroscopy* 16**,** 38-44.

Thomas, R. (2001d). Beginner's guide to ICP-MS - Part VI - The mass analyzer. *Spectroscopy* 16**,** 44-48.

Thomas, R. (2001e). Spectroscopy tutorial - A beginner's guide to ICP-MS - Part II: The sample-introduction system. *Spectroscopy* 16**,** 56-60.

Tilman, D., Balzer, C., Hill, J., and Befort, B.L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences* 108**,** 20260-20264. doi: 10.1073/pnas.1116437108.

Tremblay, G.F., Nie, Z., Belanger, G., Pelletier, S., and Allard, G. (2009). Predicting timothy mineral concentrations, dietary cation-anion difference, and grass tetany index by near-infrared reflectance spectroscopy. *Journal of Dairy Science* 92**,** 4499-4506. doi: 10.3168/jds.2008-1973.

Uddling, J., Gelang-Alfredsson, J., Piikki, K., and Pleijel, H. (2007). Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynthesis Research* 91**,** 37-46. doi: 10.1007/s11120-006-9077-5.

Ulrich, A. (1952). Physiological Bases for Assessing the Nutritional Requirements of Plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 3**,** 207-228.

Usgs. 2013. Phosphate Rock. *Mineral Commodity Summaries* [Online]. Available: http://minerals.usgs.gov/minerals/pubs/commodity/phosphate_rock/mcs-2013-phosp.pdf [Accessed February 13, 2013].

Van Der Ploeg, R.R., and Kirkham, M. (1999). On the origin of the theory of mineral nutrition of plants and the law of the minimum. *Soil Science Society of America Journal* 63**,** 1055-1062.

Van Maarschalkerweerd, M., Bro, R., Egebo, M., and Husted, S. (2013). Diagnosing latent copper deficiency in intact barley leaves (Hordeum vulgare, L.) using near infrared spectroscopy. *Journal of agricultural and food chemistry* 61**,** 10901-10910.

Villatoro-Pulido, M., Rojas, R.M., Munoz-Serrano, A., Cardenosa, V., Lopez, M.a.A., Font, R., and Del Rio-Celestino, M. (2012). Characterization and prediction by near-infrared reflectance of mineral composition of rocket (Eruca vesicaria subsp sativa and Eruca vesicaria subsp vesicaria). *Journal of the Science of Food and Agriculture* 92**,** 1331-1340. doi: 10.1002/jsfa.4694.

Wang, X.R., Wang, Y.X., Tian, J., Lim, B.L., Yan, X.L., and Liao, H. (2009). Overexpressing AtPAP15 Enhances Phosphorus Efficiency in Soybean. *Plant Physiology* 151**,** 233-240. doi: 10.1104/pp.109.138891.

Ward, A., Nielsen, A.L., and Moller, H. (2011). Rapid Assessment of Mineral Concentration in Meadow Grasses by Near Infrared Reflectance Spectroscopy. *Sensors* 11**,** 4830-4839. doi: 10.3390/s110504830.

West, M., Ellis, A.T., Potts, P.J., Streli, C., Vanhoof, C., Wegrzynek, D., and Wobrauschek, P. (2012). Atomic spectrometry update-X-ray fluorescence spectrometry. *Journal of Analytical Atomic Spectrometry* 27**,** 1603-1644. doi: 10.1039/c2ja90045a.

Westerman, R.L. (1990). *Soil testing and plant analysis.* Soil Science Society of America, Inc.

White, P. (2012). Long-distance transport in the xylem and phloem. *Marschner's Mineral Nutrition of Higher Plants***,** 49-70.

Wikstrom, F. (1994). A Theoretical Explanation of the Piper-Steenbjerg Effect. *Plant Cell and Environment* 17**,** 1053-1060. doi: 10.1111/j.1365-3040.1994.tb02028.x.

Williams, P. (2014). Tutorial: The RPD statistic: a tutorial note. *NIR news* 25**,** 22-26.

Wood, S., and Cowie, A. (Year). "A review of greenhouse gas emission factors for fertiliser production", in: *IEA bioenergy task*), 20.

Worldbank (2012). "World Data Bank".).

Yara (2013). *N-sensor* [Online]. http://www.yara.com/products_services/fertilizers/support_services/support_tools/index.aspx. [Accessed 130110 2013].

Yaseen, M., and Malhi, S.S. (2009). Differential Growth Response of Wheat Genotypes to Ammonium Phosphate and Rock Phosphate Phosphorus Sources. *Journal of Plant Nutrition* 32**,** 410-432. doi: 10.1080/01904160802660735.

Yeh, T.F., Chang, H.M., and Kadla, J.F. (2004). Rapid prediction of solid wood lignin content using transmittance near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry* 52**,** 1435-1439. doi: Doi 10.1021/Jf034874r.

Yusuf, M.A., Kumar, D., Rajwanshi, R., Strasser, R.J., Tsimilli-Michael, M., Govindjee, and Sarin, N.B. (2010). Overexpression of gamma-tocopherol methyl transferase gene in transgenic Brassica juncea plants alleviates abiotic stress: Physiological and chlorophyll a fluorescence measurements. *Biochimica Et Biophysica Acta-Bioenergetics* 1797**,** 1428-1438. doi: 10.1016/j.bbabio.2010.02.002.

Zhang, H., and Davison, W. (1995). Performance-Characteristics of Diffusion Gradients in Thin-Films for the In-Situ Measurement of Trace-Metals in Aqueous-Solution. *Analytical Chemistry* 67**,** 3391-3400. doi: 10.1021/ac00115a005.

Zheng, C.F., Jiang, D., Liu, F.L., Dai, T.B., Jing, Q., and Cao, W.X. (2009). Effects of salt and waterlogging stresses and their combination on leaf photosynthesis, chloroplast ATP synthesis, and antioxidant capacity in wheat. *Plant Science* 176**,** 575-582. doi: 10.1016/j.plantsci.2009.01.015.

Ziadi, N., and Sen Tran, T. (2008). "Mehlich 3-Extractable Elements," in *Soil sampling and methods of analysis,* eds. M.R. Carter & E.G. Gregorich. CRC Press), 107-114.

Zillmann, E., Graeff, S., Link, J., Batchelor, W.D., and Claupein, W. (2006). Assessment of cereal nitrogen requirements derived by optical on-the-go sensors on heterogeneous soils. *Agronomy Journal* 98**,** 682-690. doi: DOI 10.2134/agronj2005.0253.

Chapter 3

Spectroscopic and Chemometric Methods

Near Infrared Spectroscopy

When molecules with a dipole moment, or asymmetry, absorb light in the mid-infrared (MIR, 2500-50,000nm) part of the electromagnetic spectrum, they are excited to a higher energy state. As a result of this, they start vibrating in a number of ways. The frequencies of these fundamental vibrations determine which reflectance, or transmittance, is detected. Near infrared (NIR, 780-2500nm) spectra reflect the overtones and combination bands of the fundamental vibrations. Overtones result from excitation to higher energy states, so that wavenumbers of overtones are multiples of wavenumbers of the fundamental vibrations. Combination bands have wavenumbers of any combination of two or more fundamental vibrations, though combination bands arising from more than two vibrations are of low probability (Osborne et al., 1993;Pavia et al., 2001). Several overtones of the same fundamental vibration may occur, and there is no theoretical limit to the number of combination bands in a spectrum. This results in a high number of overlapping, non-specific peaks, which make the NIR spectrum an indistinct picture of all IR-active chemical constituents in the given sample. In addition to these effects, hydrogen bonds occur. They are weak bonds between H and mainly O or N, which affect the vibrations of the molecules involved and thereby cause peak shifts, observed as peak broadening. An example of NIR absorption spectra from barley leaves is presented in Figure 1. Where a univariate data analysis to some extent may work in MIR, the broad and overlapping peaks in NIR mean that data analysis here depends on multivariate methods (Næs et al., 2004).

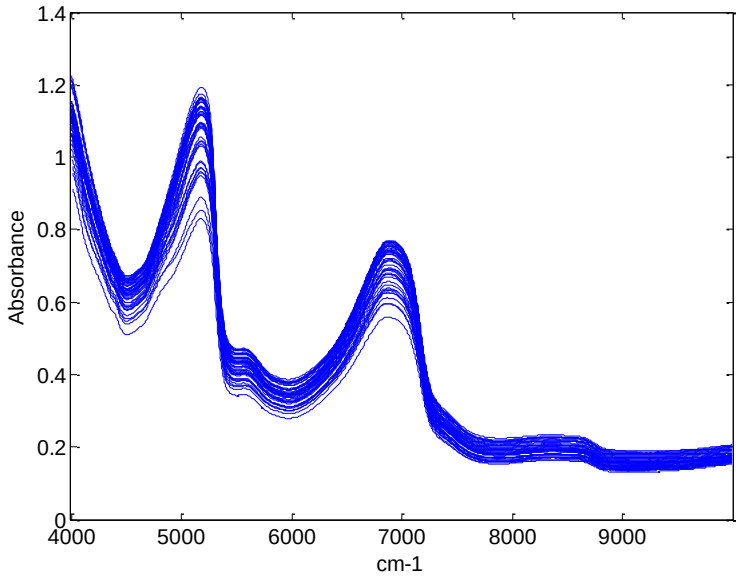

Figure 1. NIR absorbance spectra measured on the adaxial side of barley leaves. The x-axis shows wavenumbers in cm^{-1}, and the range from 4000 – 9000cm^{-1} is equivalent to 2500 – 1111nm. Spectra are a subset of data from Paper II.

Wavenumbers, with the unit cm^{-1}, are a commonly used x-axis unit for NIR spectra because using these, overtones and combination bands are directly calculated as multiples or sums of fundamental vibrations. Those calculations are less simple when based on wavelengths, usually measured in nm, though the unit is often encountered in NIR literature. Which unit is used seems to depend on the type of instrument employed. The look of spectra will change when switching between units, but data, and thus results of data analysis, are the same.

Actual measurements in NIR are typically recorded as reflectance, as in Paper II, or as transmittance, but a different unit, commonly denoted "absorbance", is usually employed in the subsequent analysis. It is related to reflectance as:

$$A = \log\left(\frac{1}{R}\right) = -\log(R) \qquad (1)$$

Where *A* is "absorbance" and *R* is the detected reflectance (Geladi et al., 1985). Actual absorbance is calculated as the negative logarithm of transmittance, but the "absorbance" measure presented in equation (1) is likewise linearly related to concentrations of absorbing species, according to Beer's law (Harris, 2007). Thus, for reflectance measurements the "absorbance" unit is convenient for calculations, though correctly it should be, and sometimes is, denoted log(1/R).

The analysis of the broad and overlapping peaks in NIR spectra is further complicated by interferences from variation in factors such as sample thickness, refractive index and particle size as well as light scattering (Geladi et al., 1985;Næs et al., 2004). The occurrence of such can be limited by ensuring uniform sample presentation and handling and optimization of instrumental settings and the recording of a blank background spectrum. In the reflectance measurements used in Paper II, this background was a completely white object. Including the background measurement in equation (1) results in the following:

$$A = -\log\left(\frac{I_{sample}}{I_{background}}\right) \qquad (2)$$

Where A is absorbance, and I_{sample} and $I_{background}$ are the spectra of sample and background recordings, respectively. However, precautions during sample handling cannot remove all light scattering, which may cause both additive and multiplicative effects and results in a lack of linear relation between absorption and species concentrations. Fortunately, however, the noise can be corrected for, and doing so before analyzing a data set enables the development of a simpler and thus more accurate model.

Multiplicative Signal Correction

The NIR spectra in Paper II were preprocessed using Multiplicative Signal Correction (MSC), a common method for preprocessing of NIR spectra (Næs et al., 2004). Multiplicative signal correction is a method developed for removing additive and multiplicative noise from spectra, based on the fact that the dependency of light scatter on wavenumber is different from that of chemically based light absorption (Geladi et al., 1985). By using a number of measurements it is therefore possible to ensure a uniform contribution of light scatter to the measured absorbance of all samples. The MSC is an object-wise correction, meaning that spectra from each measurement are corrected one at a time. The model for each individual spectrum is:

$$\boldsymbol{x} = a + b\bar{\boldsymbol{x}} + \boldsymbol{e} \qquad (3)$$

Where $\boldsymbol{x}$ is a spectrum, a is the average additive effect, b is the average multiplicative effect, and $\boldsymbol{e}$ is the residual, which optimally represents the chemical information in $\boldsymbol{x}$. An "ideal" sample is represented by $\bar{\boldsymbol{x}}$. The MSC preprocessing of each sample takes place by first subtracting a (equivalent to intercept) from each sample at each wavelength. The result of this is divided by b (equivalent to slope). The spectra presented in Figure 2 are the same as were shown in Figure 1, only after MSC preprocessing, to demonstrate the effect.

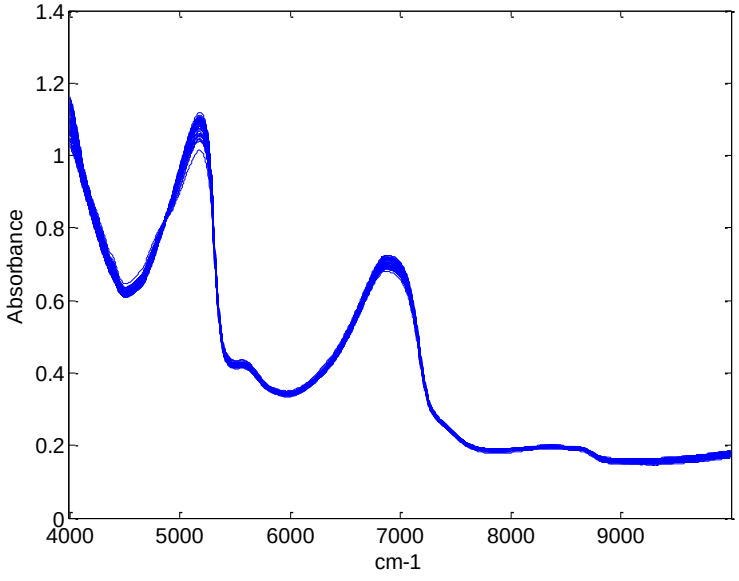

Figure 2. Plot of NIR spectra from Figure 1 after MSC preprocessing.

In practice, the "ideal" sample will usually be the average spectrum of the dataset, which makes MSC sensitive to outliers. This is a major weakness of the method, making thorough outlier removal extremely important. Alternatively, a median spectrum or a differently selected "ideal" sample may be employed.

Chlorophyll *a* Fluorescence

In fluorescence spectroscopy, emission of light is measured during relaxation of molecules excited by incoming light. Actinic light reaching a photosynthetically active tissue is used either for photosynthesis, reflected directly or re-emitted as chlorophyll fluorescence. During the first second of emission of chlorophyll *a* fluorescence, a characteristic curve called the Kautsky curve or OJIP transient, after the four, characteristic plateaus it develops, is formed (Figure 3).

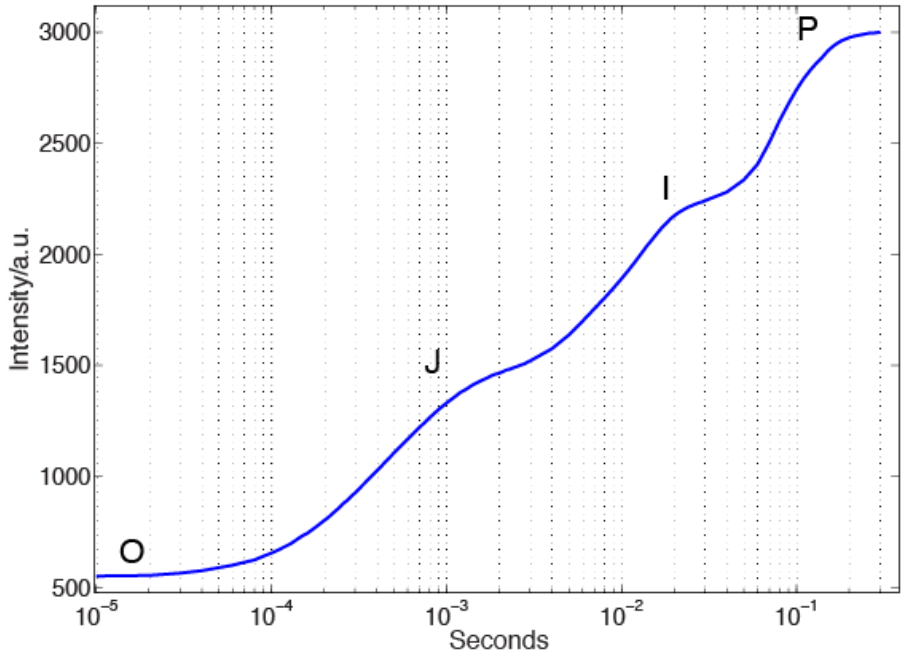

Figure 3. Kautsky curve or OJIP transient of a healthy barley leaf. The four plateaus, O, J, I and P, are indicated in the figure.

Before recording chlorophyll *a* fluorescence, the leaf is dark adapted for 20 minutes to render all photosystems in the open, or oxidized, state. The O plateau, or F_0, indicates the level of background fluorescence, and this is determined by exposing the dark-adapted leaf to a short pulse of non-actinic light before the actual measurement. Gain of the detector is adjusted accordingly to avoid saturation. The leaf is then exposed to actinic light at a high intensity (>3000µmol m^{-2} s^{-1}), and after the first 0.3 seconds of this, the maximal fluorescence intensity, the P plateau of the OJIP transient, is reached. Exciting light is optically filtered to a maximal wavelength of 650nm, and likewise, another optical filter ensures that only light of wavelengths longer than 650nm reaches the detector, ensuring that only actual fluorescence, and no reflections from exciting light, is detected.

The exact mechanisms resulting in the various plateaus of the OJIP transient are not yet fully understood, though it is generally accepted that the OJIP transient reflects events in the photosynthetic electron transport chain, and that the J, I and P plateaus are assumed to be caused by kinetic bottlenecks (Schreiber et al., 1989;Schansker et al., 2005;Stirbet and Govindjee, 2011). Analyzing OJIP transients is often done using the so-called JIP test. Rather than an actual test, this is a wide range of parameters calculated from only 5-6 data points and corresponding areas above the OJIP transient (Force et al., 2003). The simplicity is a great advantage of the JIP test and a good reason for the widespread use of it. Parameters from the JIP test have proven useful *e.g.* as parameters describing general photosynthetic performance (Force et al., 2003;Swain et al., 2010) and the F_v/F_m specifically for diagnosing latent Mn deficiency (Hebbern et al., 2005). However, as the JIP test is based on specific points and not the whole course of the OJIP transient, correlations may be overlooked. The work for the present thesis clearly demonstrates this, as the impact of P deficiency on OJIP transients has in fact been investigated previously (Ripley et al., 2004;Jiang et al., 2009;Lin et al., 2009). However, focusing on the parameters defined in the JIP test instead of the complete course of the OJIP transient means that the systematic correlation between P nutrition and the shape of the OJIP transient, as identified in Papers III and IV, was overlooked.

OJIP transients were preprocessed using an approximation to differentiation, being the difference between consecutive measuring points (Papers III and IV). This preprocessing emphasizes the shape of the curve, thereby enabling subsequent multivariate data analysis to focus on this rather than absolute intensities.

Multivariate Data Analysis

For analysis of spectroscopic data, multivariate methods, chemometrics, are now the most commonly used, for obvious reasons. As discussed, the peaks in NIR spectra are broad and represent only an indistinct image of the fundamental vibrations. To identify general patterns, it is therefore necessary to include information from the whole spectrum and a relatively large number of samples. Even in spectra with more discrete peaks, such as MIR, including a broader range of data will often add robustness to a calibration, though larger amounts of irrelevant data still have to be avoided (Kjeldahl and Bro, 2010).

Generally, chemometric methods can be divided into quantitative and qualitative analyses, where qualitative analyses count both supervised and unsupervised methods. Principal component analysis (PCA), partial least squares analysis (PLS) and partial least squares – discriminant analysis (PLS-DA) are employed in Papers II – IV and are, thus, further described here.

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. It is often used for unsupervised exploration of samples in a data set. The data matrix, $\boldsymbol{X}$, is approximated using the least squares model in equation (4):

$$\boldsymbol{X} = \boldsymbol{TP} + \boldsymbol{E} \qquad (4)$$

T is a score matrix, ***P*** is a loading matrix and ***E*** is the residual. ***X*** is often mean centered, meaning that the average value of each variable is subtracted from data, or autoscaled, where mean centered data are furthermore divided by the standard deviation of the variable. Mean centering causes a shift, which in most cases improves the presentation of results. Autoscaling centers data and in addition enables the use of variables of different scales, such as *e.g.* shoot biomass in grams and root length in centimeters.

Geometrically, the PCA finds a set of new axes, named loading vectors, for ***X***. The loading vector and its associated score vector is called a principal component (PC). The direction of the first loading is coincidental with the largest variation in ***X***. The second loading is orthogonal to the first and represents, given the restriction of orthogonality, the second largest variation in ***X,*** and so on. Data is visualized on these new information-rich axes, where often the first few will be sufficient for showing major differences between samples. Especially for spectroscopic data, where the covariance between variables is very high, the PCA offers a significant reduction of dimensions, often down to 3-4 PC's, and thereby enables identification of sample clustering, which may not be easily seen from the raw data (Wold et al., 1987;Næs et al., 2004).

Partial Least Squares Analysis

The PLS analysis is a method for quantitative prediction that models the relationship between a data matrix, **X**, *e.g.* a set of spectra, and a response matrix, **Y**, as well as the structures of **X** and **Y**. In the present work, only PLS1, which uses a response matrix containing *one* column, *i.e.* a vector **y**, is used. In the present work, the response vector represents nutrient concentrations in leaves.

Geometrically, the explanation of the PLS regression has similarities to that of PCA, and the data matrix is described by scores and loadings like the PCA, see equation (4). In addition, **y** is described by the scores matrix, **T**, loadings, **q** and residuals, **f**:

$$\boldsymbol{y} = \boldsymbol{Tq} + \boldsymbol{f} \qquad (5)$$

In PLS, the first loading weight vector, or latent variable (LV), of **X** is determined not to describe the major variation in **X**, as in PCA, but to maximize the covariance between **y** and **X.** This is done by rotating the first loading vector in the **X** space to the optimal position, where the residual in a least squares regression of **X** scores against **y** is minimized. The second LV is determined using the same methodology, only **X** and **y** input is now based on residuals calculated by subtracting the first component from the original data matrices, and the process is repeated for subsequent LV's until the desired number of components has been extracted. The final outcome of the PLS regression is, thus, a model which is able to predict **y** values based on **x** input (Geladi and Kowalski, 1986;Wold et al., 2001;Næs et al., 2004).

Partial Least Squares – Discriminant Analysis

The PLS-DA is a supervised classification method, which is basically a special case of the PLS analysis. In PLS-DA, **y** is a dummy matrix containing 1 and 0, or -1 and 1, referring to whether or not a given sample belongs to a class. A PLS calibration is then carried out, and the loading weight vectors will, according to the description above, be orientated in the direction of **X** that

maximizes the separation between samples belonging to the given class in **y** or not (Wise et al., 2006).

Both PCA and PLS-DA can be used to envisage clustering of samples. A main difference is, however, the subject of supervision. The PCA is a "pure" method that visualizes sample distribution based exclusively on **X** input. It gives a clear overview of what can be concluded from data alone, presupposing no prior knowledge of samples. The PLS-DA on the other hand is supervised, meaning that input regarding the class of calibration samples is provided. This enables the model to focus on the information most relevant for classification and hence, often use fewer components (LV's) to obtain the result (Barker and Rayens, 2003;Næs et al., 2004). Precaution is however a prerequisite. If no validation or cross-validation is performed, the apparent result of the model will be overly optimistic. Predicting the class of samples while already knowing the class is obviously always possible, irrespective of the true correlations (Kjeldahl and Bro, 2010).

Chapter 4

Sampling and Experimental Variability

Working with live plants is asking for trouble. A batch of seeds may suddenly experience an almost complete loss of viability, plants can be attacked by pests and though cultivation is done according to a set scheme, deviations in growth effects are expected. Substantial deviations usually have a cause, though, and in such cases, plant scientists have the advantage of being allowed to "kill the patient" and improve the setup in a successive experiment. However, a natural variation will always occur between individual plants, even within genotypes. When basing experimental work on such material, it is important to investigate how much of the variation in the experiment derives from random factors such as growth units and plants, and how much from the actual treatments. This provides insight into which performance level can be expected from calibration work, as a large influence from random factors hampers the identification of systematic effects.

The experimental work of the present thesis was initiated as a screening of various methods and their ability to predict different nutrient deficiencies in plants. A number of spectroscopic methods were employed, and the two most successful findings resulted in Papers II, III and IV, employing NIR and chlorophyll *a* fluorescence. The contribution of random factors to the total variance within these measurements is examined more closely here to support the overall validity of the results.

Near Infrared Spectra of Leaves

Within Leaf and Within Plant Variability

A batch of barley plants were cultivated in hydroponics, according to the method described in Paper II, and NIR spectra were recorded after four weeks, when deficiency was established in Cu-deprived plants. The within-leaf variation was investigated by measuring three different places in one leaf; on the middle of the lowest (basal), middle and apical (apex) thirds of the leaf. The positions are illustrated in Figure 4.

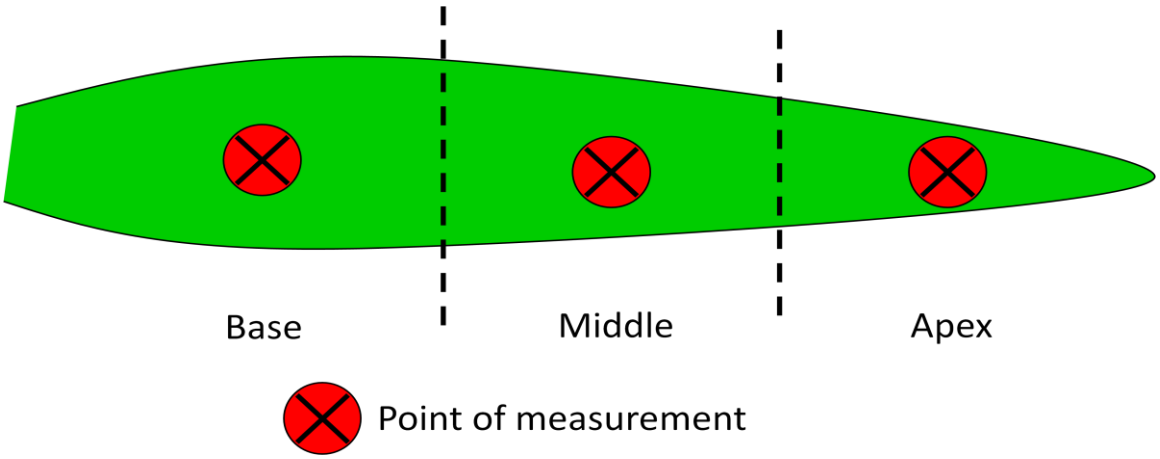

Figure 4. Positions of NIR measurements within a barley leaf, for investigation of within-leaf variability.

Investigating the preprocessed (MSC and mean centered) data revealed that especially "apex" samples deviated from the "middle" and "base" samples and showed great variance within the group as well (Figure 5a). Performing a PCA on the same data confirmed this observation (Figure 5b).

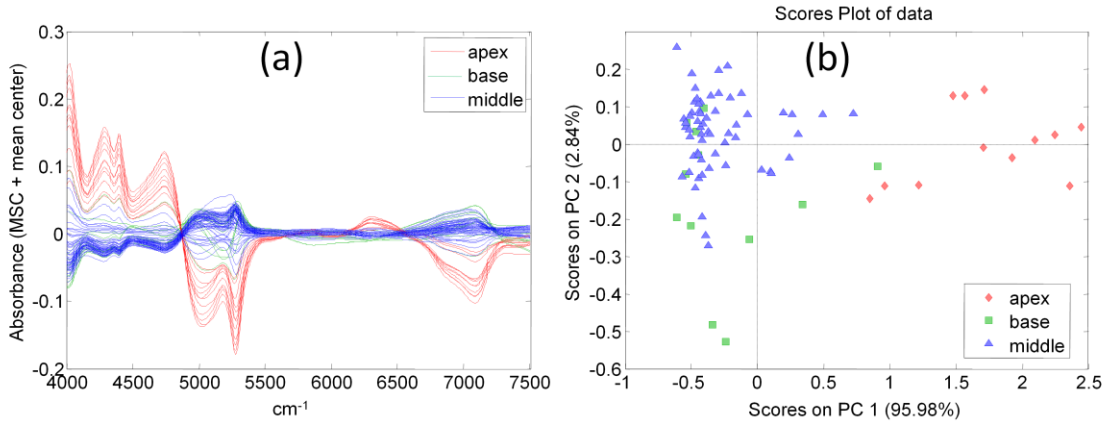

Figure 5. Preprocessed (MSC + mean centering) NIR spectra of barley leaves measured in three different leaf positions (a) and scores plot of a PCA on the same NIR spectra (b). Both reveal "apex" samples as deviating strongly from "base" and "middle" samples. In (b), the separation is along PC1, which explains 96% of the total variation in data, demonstrating the high degree of deviation of "apex" samples.

Part of the "base" samples deviate along PC2 from the main group of "middle" and "base" samples. However, as PC2 accounts for less than 3% of the total dataset variation, this spectral deviation is less significant than that of "apex" samples, which is also seen in the preprocessed spectra (Figure 5).

Primary plant growth occurs at the apical meristems, meaning that the apex is the youngest part of the leaf (Raven et al., 1999). All measurements were carried out on the so-called "youngest fully developed leaf" (YFDL) of plants, however, these measurements imply that the term "youngest fully *expanded* leaf" would be more accurate, as the deviation of the apex clearly that compositional changes are ongoing and that this part is not yet fully developed in all leaves, though the leaf blade has indeed fully expanded.

The “apex” measurements along with “base” measurements were accordingly removed from the dataset. To avoid any uncertainties in future data analysis, all spectra recorded after this point, including those used in Paper II, were therefore derived from the middle of the leaves.

Measuring a completely homogeneous sample repeatedly using NIR should theoretically yield identical spectra, if no degradation of NIR active tissue components or metabolites takes place and heating effects are avoided. Heating effects occur if a sample is measured repeatedly without removing it from the instruments in between measurements. In a PCA scores plot, heating is recognized as a gradual displacement of the sample, moving in one specific direction, as repeat number increases. However, leaves are far from homogeneous, with an irregular surface and the presence of veins. In a minor study, five samples were measured five times each, immediately after harvesting from the plant. The middle of the leaf was found, measuring was performed, and the leaf was removed from the instrument. Again, the middle was found, the leaf was placed and measured, and removed. No heating effect occurred, and a scores plot of a PCA on the data (preprocessed by MSC and mean centering) along with the remaining samples of the study, which were measured only once, is found in Figure 6.

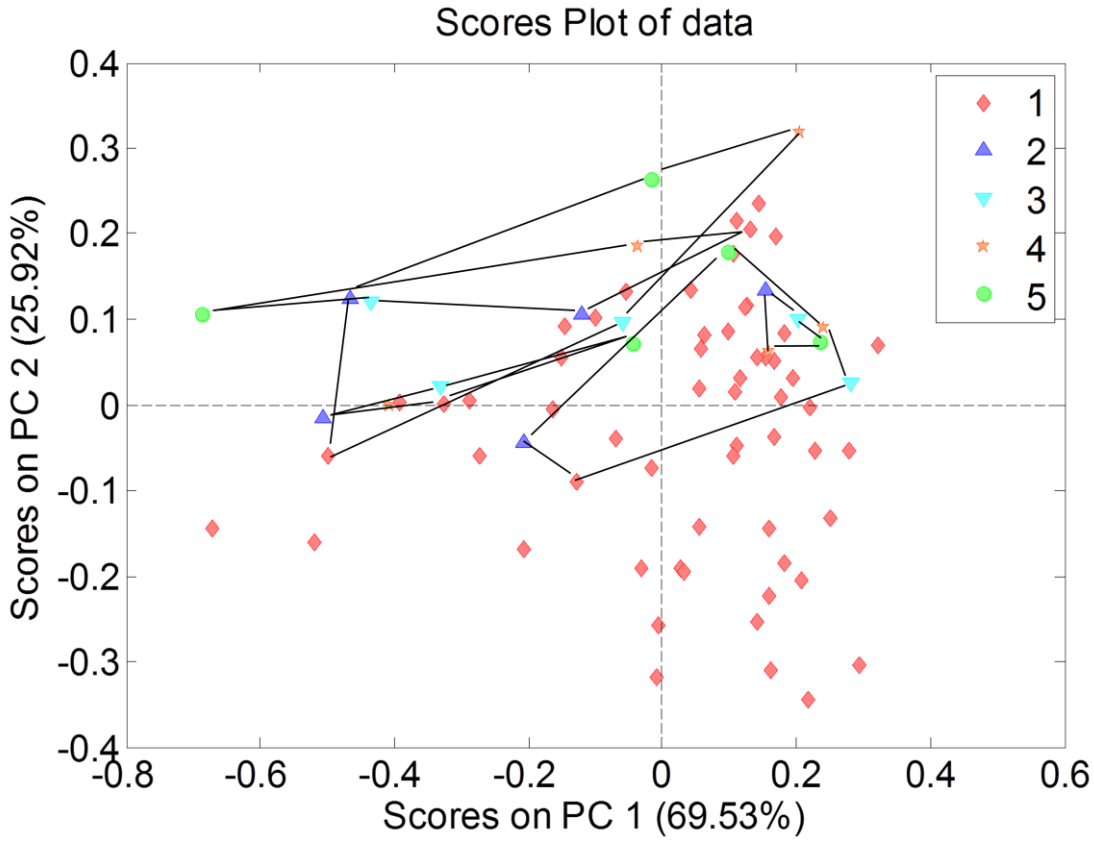

Figure 6. Scores plot of a PCA indicating the variability of measuring the same leaf samples 5 times in repeat. 95% of total variation in the dataset was explained by the first two PC's, presented in the figure. Repeated measurements are connected with lines; repeat number for each measurement is indicated in the figure. Remaining samples in the PCA are measured only once. Repeated measurements do not follow a specific direction, meaning that no systematic effect of repeat number is found to be of high importance.

No systematic effect of the repeat number is seen, *i.e.* there is no specific grouping or direction of the repeats, meaning that no systematic degradation of tissue or metabolites within these

first, few minutes after harvest is indicated. However, it is evident that identical measurements from each leaf are absolutely not obtained, despite the identical procedure employed, and this constitutes a fundamental weakness of any method using NIR spectra derived from fresh barley leaves, or supposedly fresh leaves from any other plant species. Cu deficiency was not induced at an NIR-detectable level at the time of measuring, why all samples concerned must be regarded as control samples. To minimize the problem, several spectra could be collected from each sample, and the average or median spectrum used for analysis. This would, however, require equipment fitted more specifically for this purpose to enable sampling of a high number of leaves within a reasonable time frame.

Variability of Hydroponic Growth Units

Estimating the variability between hydroponic growth units is measuring the success of repeating the exact same conditions for plants cultivated in different units but provided in theory the same conditions. This needs to be done in a larger study, including many units, and simply investigating PCA scores plots will not provide the sufficient overview, as various effects of treatments need to be taken into account as well.

Linear mixed models (proc mixed command in SAS version 9.3, SAS Institute, USA) is a method that estimates the contribution and variance of random factors as well as the contribution of systematic factors to the total variance in the data set. It has the general formulation:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{Za} + \boldsymbol{e} \qquad (5)$$

Where $\boldsymbol{y}$ is the vector of observations, $\boldsymbol{b}$ is the vector of systematic effects, $\boldsymbol{a}$ is the vector of random effects and $\boldsymbol{e}$ is the vector of residual error. $\boldsymbol{X}$ and $\boldsymbol{Z}$ are incidence matrices that relate observations to systematic and random effects, respectively (Hofer, 1998). In the present investigation, the only random effect that can be assigned specifically is the variation between hydroponic growth units. Systematic effects derive from nutrient level, day of measuring, due to progressing nutrient deficiency, and for the P experiment also the climatic treatment, as plants were cultivated under different light and temperature settings (see Paper III). The error term will include variation between plants within a growth unit and measuring error. As each sample is only measured once, these terms will be confounded and therefore cannot be separated. The observations vector, $\boldsymbol{y}$, can contain score values from a selected latent variable of a PCA or PLS based on a relevant dataset. The predictions of variance for systematic, random and residual effects are joined to obtain the relative contribution of each for comparison.

The NIR measurements of the time series of progressing Cu deficiency (Paper II) were used as a basis for the investigation of variability in NIR spectra between growth units. A PCA was carried out on the complete dataset, with preprocessing, MSC and mean centering, as described in Paper II. Where PC1 mainly related to the age of plants, PC2 showed a clear effect of increasing Cu deficiency (Figure 7).

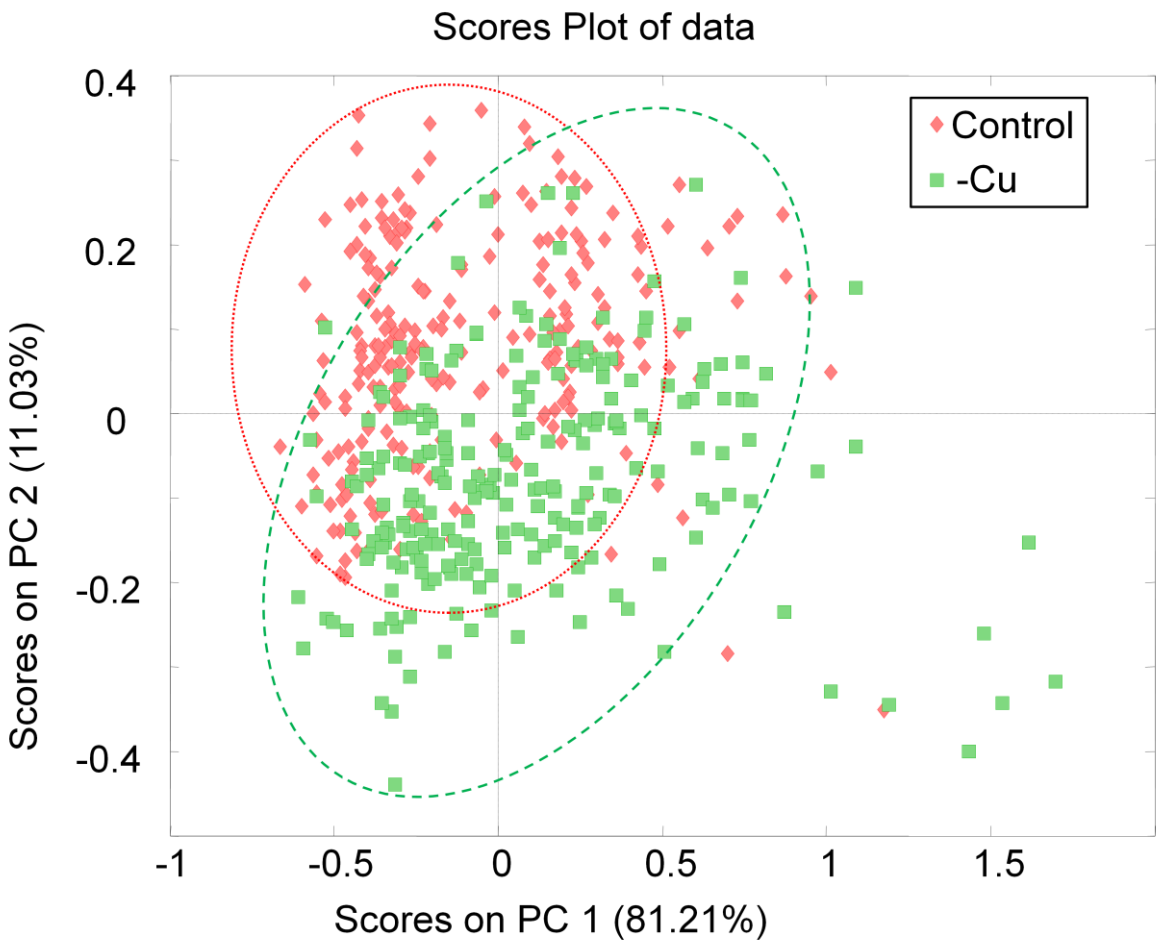

Figure 7. Scores plot of a PCA based on preprocessed (MSC + mean centering) NIR spectra from the time series of progressing Cu deficiency (Paper II). 92% of total variation in the data set is explained by the first two PC's. Plant age and degree of Cu deficiency interact, as Cu deprived plants go from healthy to deficient over time, why the separation between treatments is not completely clear in this figure.

The output of the statistical analysis is found in Table 1, where it is seen that systematic effects accounted for 55 and 74%, respectively, of the explained variability in PC1 and PC2, and only 3 or 2% could be ascribed to growth units. The residual error term contributed with 42 and 24%. In this case, error includes the variation between plants within a growth unit, which are subject to competition, and measurement error. In practice, measurement error will to some degree affect other terms in addition to residual error, but the larger the number of samples, the less this effect.

Table 1. Total and relative contribution of systematic and assigned random (i.e. growth unit) effects as well as error to the explained variability in PC1 and PC2 scores. Together, 92% of the total variation in data is explained by PC1 and PC2.

	PC1		PC2	
	Variance	**% of total**	**Variance**	**% of total**
Systematic	0.094	55%	0.039	74%
Growth unit	0.005	3%	0.001	2%
Error	0.072	42%	0.013	24%

The contribution of random error is almost twice as big when analyzing PC1 scores compared to PC2 scores (Table 1). PC1, as stated, is for a large part spanned by sample age, and from

Figure 5 it was derived that spectra were highly influenced by which part of the leaf blade was sampled or, in other words, the age of the sampling area. Whereas plant age, as measuring day, is included in the systematic part of the mixed linear model, the exact leaf age is not known and may vary from "just unfolded" to right before the next, younger, leaf is completely unfolded. Age of the sampling area is therefore very likely to be a main contributor to the relatively large random error, especially in PC1. The high variability that was observed between repeated measurements on the same leaf following the exact same procedure (Figure 6) is another contributor to random error in both PC1 and PC2 and must be regarded a basic weakness of recording spectra in NIR, and possibly other wavelength ranges, on fresh leaf samples.

In spite of the large contributions of random error, it is demonstrated in Paper II and numerous other publications that correlations can in fact be found between NIR spectra and plant nutritional status (see Paper I for review), though in many cases, correlations are poor (*e.g.* Dealdana et al. (1995);Cozzolino and Moron (2004);Liao et al. (2012)). Measuring a large number of samples will generally increase robustness and to a higher degree enable models to find characteristics in the spectra related to treatment, despite the random error. Employing PLS-DA instead of PCA might also, as demonstrated in Paper II, improve the classification power for the NIR-based model. Finally of course, there will be cases where no fingerprint of a nutrient deficiency can be found in corresponding NIR spectra.

Chlorophyll *a* Fluorescence Measurements

The contributions to total dataset variation from systematic and random effects as well as from error were investigated for chlorophyll *a* fluorescence measurements, in the form of OJIP transients, in a similar way as for NIR data. No measurements to support a study of the effects of measuring position within the leaf were carried out, nor was repeatability of measurements in a single leaf investigated, why the contribution of these factors to the random variation cannot be explored.

OJIP transients were recorded in the YFDL and 2nd YFDL of plants in experiment 2 of Paper III. A PCA was carried out on the differentiated and mean centered transients, *i.e.* a preprocessing similar to that employed in Paper III, and included control samples and three levels of increasingly P deficient samples, measured at three different measuring days. The scores plot of the first two PC's is presented in Figure 8.

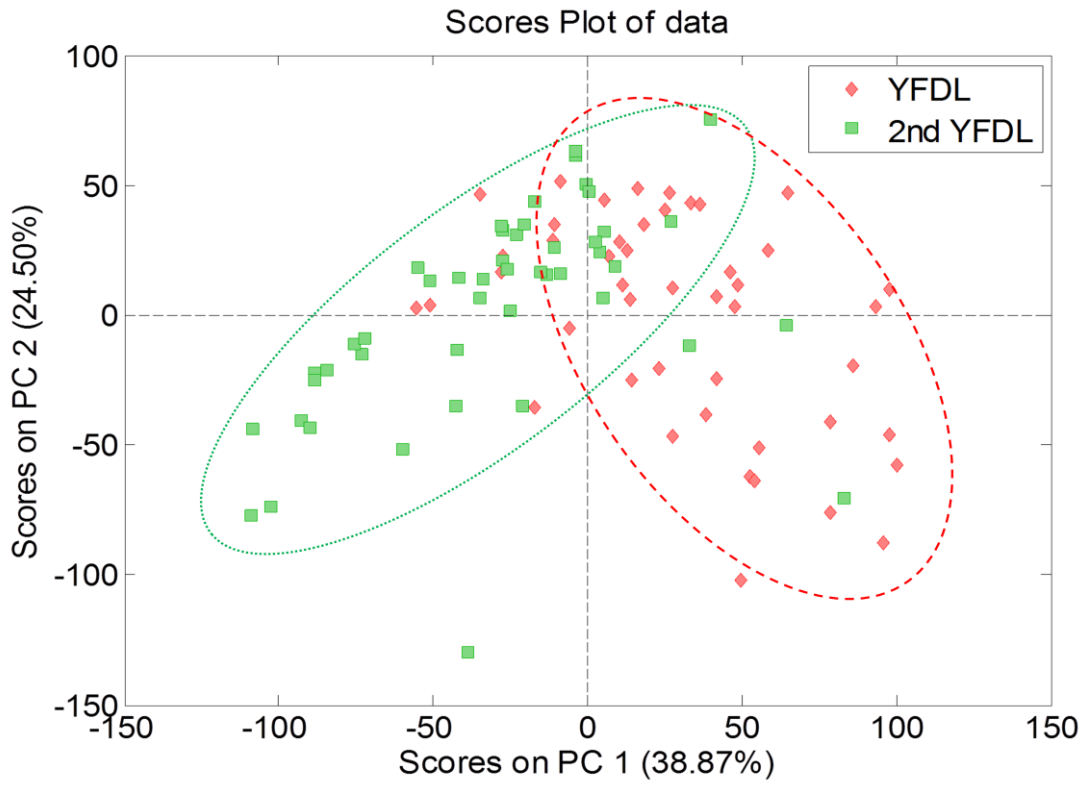

Figure 8. Scores plot of a PCA based on differentiated and mean centered OJIP transients measured on YFDL and 2nd YFDL in barley plants. The first two PC's, explaining 63% of total dataset variation, clearly show systematic differences between the two leaf ages. Data from Paper III.

As a highly phloem mobile nutrient, retranslocation of P from the older parts of the plant to the youngest parts takes place, and the general difference found between YFDL and 2nd YFDL was therefore expected. Inspecting the differentiated OJIP transients reveals that around the I-step, which is the main part of the transient affected by P deficiency, values of transients measured on the 2nd YFDL are at a lower level than transients from YFDL, for both control and P deficient samples. Structural differences between YFDL and 2nd YFDL, such as chlorophyll concentrations, are likely to cause this difference. As demonstrated in Paper III, applying a model developed on YFDL to 2nd YFDL samples does not work, and hence, settling on one specific leaf number is necessary. Choosing the 2nd YFDL for measuring purposes could be an advantage in terms of early detection of deficiency. However, the frequency of physiological spots and other minor damages on the 2nd YFDL's was found to be rather high, why the YFDL was preferred for the analytical work.

To investigate the relative contributions of systematic and random effects to the total dataset variance, mixed linear models were employed in a procedure similar to that for NIR spectra. A PCA was based on the dataset from experiment 1, Paper III, after differentiation and mean centering. The scores of PC1 and PC2 represent almost 80% of variation in data (Figure 9), and it is seen that PC1, explaining 61%, mainly reflects P concentrations. Thus, scores of PC1 were chosen as input for the statistical analysis.

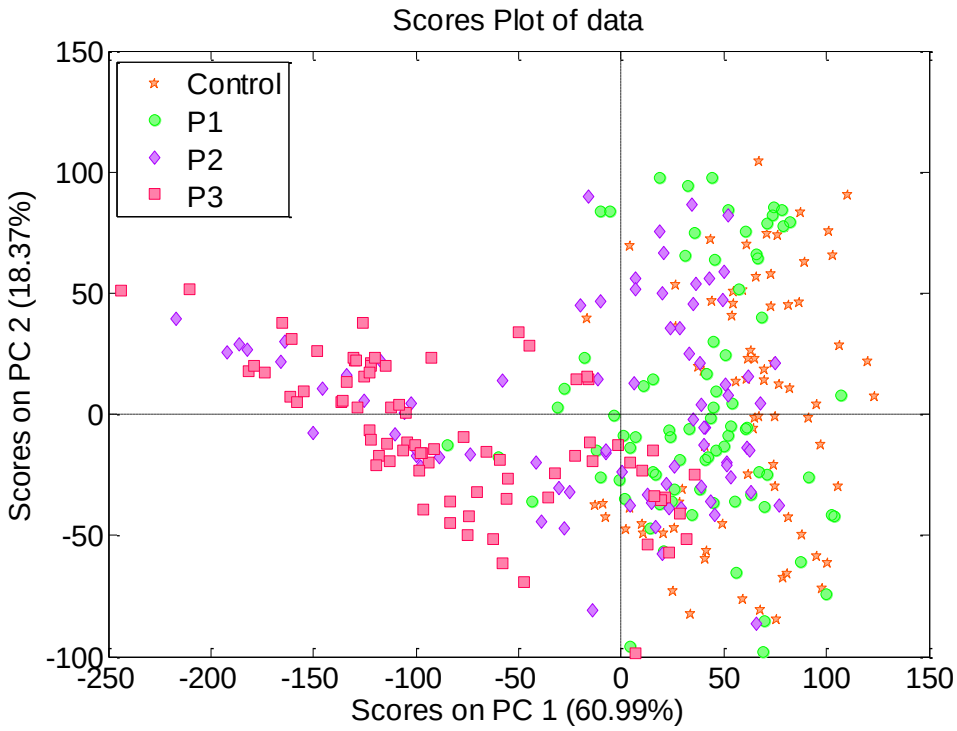

Figure 9. Scores plot of a PCA based on differentiated and mean centered OJIP transients. More than 79% of total dataset variation is explained by the first two PC's. P1, P2 and P3 treatments represent decreasing levels of P provided to plants. P3 samples are P deficient both measuring days, whereas P2 and to a lesser extent P1 go from healthy to P deficient between the two measuring days. This is reflected in the scores plot by samples moving diagonally, roughly in the direction from 1st to 3rd quadrant. Data from Paper III (experiment 1).

It is noted that the P3 plants, the most P deficient, and to a lesser degree also P2 plants have a large within group deviation along PC1. For P2 this regards mainly samples from the second measuring day, when P deficiency was most pronounced. Control and P1 plants have less within group variance along PC1. Phosphorus deficiency in plants is induced gradually when P supply is limited. As P becomes scarcer, the competition between plants increases and, thus, results in larger variation within growth units. Where P3 plants were clearly P deficient already the first measuring day, P2 plants moved from less to more pronounced P deficiency between measuring days 1 and 2, why the variation between individual plants also increased.

Table 2 shows the result of the statistical analysis. It is seen that growth units contribute with almost no variation in the dataset. This is a very positive result, indicating once again that conditions in different hydroponic units are very similar when provided the same treatments. Residual error contributes with as much as 28% of the total explained variance, which is a lot. This error includes variation between plants, within leaf variation and measuring error.

Table 2. Total and relative contribution of systematic and assigned random (growth unit) effects as well as error to the explained variability in PC1 scores. PC1 explains 61% of total dataset variation.

	Variance	% of total
Systematic	4450	71.2%
Growth unit	21	0.3%
Error	1777	28.4%

The large variation observed within P2 and P3 treatments is likely to explain the significant size of the error term, if the variation is found within each bucket. This was further investigated by calculating the variance of PC1 score values within each bucket, averaging these values for treatments (P-level * Chamber * Day) and finally take the square root for comparison of average standard deviations. The results are presented in Table 3. Relatively low values (<30) are found for all control treatments, P1 treatments with one exception, P2 treatments on the first measuring day and for no P3 treatments. The highest values are found for P2 treatments on the second measuring day. This supports the observation of large variation within P2 and P3 treatments, and that these are likely to be major contributors to the large residual error of the mixed linear models.

On the second measuring day in cold climate treatment, the average standard deviation for P3 plants was significantly lower than the three other results for P3 treatment. The severe degree of P deficiency in all plants in the growth unit at this stage might explain this lower level of variation, as all plants will be critically impaired by the deficiency.

Table 3. The average standard deviation within buckets for each combination of systematic effects, meaning P-level, chamber (climatic treatment) and measuring day.

P-level	Chamber	Day	Avr. Standard Deviation
Control	Warm	1	23.1
Control	Warm	2	16.3
Control	Cold	1	26.5
Control	Cold	2	25.6
P1	Warm	1	19.2
P1	Warm	2	48.0
P1	Cold	1	26.8
P1	Cold	2	26.4
P2	Warm	1	15.5
P2	Warm	2	78.6
P2	Cold	1	16.0
P2	Cold	2	80.0
P3	Warm	1	51.5
P3	Warm	2	56.0
P3	Cold	1	50.5
P3	Cold	2	31.0

When P nutrition becomes scarce, competition between plants is expected. A slight difference between plants in *e.g.* root biomass can in such a case have major influence on the share of the limited P that each plant is able to take up. Rapidly this will increase the differences between plants even more, and a large deviation within one growth unit occurs. It is indicated in Table 3 that the large variability between plants occurs when nutrient levels decline and to a lesser degree when sufficient or only slightly limited P supplies are provided. The large error term is, thus, partly due to a biological competition.

From the analysis of NIR spectra and OJIP transients it is concluded that the experimental methods employed were highly valid, as the main parts of total dataset variation derived from either systematic effects, *i.e.* treatments, or from random variation, which for a large part can be explained. The variability within leaves and between repeated measurements of OJIP transients is an obvious subject for further investigation, which would be of general interest for investigations of factors influencing chlorophyll fluorescence in plants. If using NIR to measure on fresh leaf material, improving the repeatability of sampling would decrease method error significantly. This could be done by optimizing sampling equipment for leaf analysis, enabling a number of consecutive measurements to mathematically “even out” the inhomogeneity of leaves. Improving the raw spectra this way would be expected to improve results of existing methods and may as well increase the possible applications.

Chapter 5

Conclusions and Perspectives

The aim of this thesis was to investigate whether latent deficiencies of essential plant nutrients can be detected by common spectroscopic techniques, using barley as a model plant. The work was initiated by methodological screening experiments, ending up in the development of two specific methods. The main conclusions are:

- Near Infrared Spectroscopy can be used to distinguish between Cu deficient and Cu sufficient barley leaves. The method is specific for Cu, and the deficiency can be detected already at a latent stage.
- A quantitative assessment of Cu concentrations using NIR results in high prediction errors.
- Lignin concentrations are unaltered when Cu deficiency is first detected using NIR. Thus, decreased lignin concentration is not the first effect of Cu deficiency as commonly stated in the existing literature.
- Near Infrared spectra of leaf samples are clearly affected by age and measuring position on the leaf.
- Repeated NIR recordings on the same leaf show large variation. Consequently, several repeated measurements will be required to increase analytical precision.

- Latent P deficiency is reflected in OJIP transients, with the main effect being a marked straightening of the I-step. The effect is specific for P deficiency.
- Using OJIP transients, P concentrations in deficient plants can be assessed quantitatively.
- The same effect of P deficiency on OJIP transients is found in tomato, indicating a mechanism affecting plant photosynthesis in a general manner.
- Indications of specific effects in the OJIP spectra for deficiencies of S, Mg, Cu and Fe are found, in addition to the already known effect of Mn deficiency.

- Variability between hydroponic growth units exposed to similar experimental treatment is very low for both NIR and chlorophyll *a* fluorescence measurements.
- At deficient nutrient levels, a significant competition between plants within a hydroponic growth unit occurs.

Nutrient management in agriculture is as relevant as ever, though the character of the problems has large spatial variation. In Europe and North America, agricultural practice is very intensive, and environmental concerns are prevailing, with good reason. Methods to assist in optimal nutrient management are highly needed to maintain the high soil fertility and ensure optimal yields, while at the same time protecting the environment and cutting energy consumption from production and distribution of fertilizer. In China, mineral fertilizer along with livestock production have been identified as the major contributors to the deterioration of surface water quality (Chen et al., 2008), and optimization of nutrient management is therefore a major challenge. Good agricultural practice is a prerequisite for making the optimal use of even the most advanced, automated instruments and methods for assisting plant producers. But to optimize fertilizer management, assistance from analytical methods is crucial. This thesis demonstrates clearly that spectroscopic methods have huge potential for fast, cheap and accurate assessment of plant nutritional status.

Spectroscopy to detect nutritional disorders is a little investigated field, with the exception of Vis-NIR, leaving many opportunities open. Further screening of methods and nutrient deficiencies is likely to reveal even more correlations, of which a number have been indicated during the work behind this thesis. In addition to detecting latent Cu deficiency using NIR (Paper II), many papers on prediction of a range of other, essential plant nutrients using NIR or Vis-NIR are found. Though not as thoroughly investigated previously, there are indications in this thesis that OJIP transients could be able to predict deficiency of a number of nutrients in addition to P. Assessing status of several nutrients using only one method or device would be a huge advantage, saving time during measuring as well as providing plant producers with a more complete assessment of plant nutritional status. This would be a great advantage for optimization of fertilizer management.

The aim of fertilizer management can be condensed into assessing the right time, the right rate and the right place of fertilizer application. Whereas placement, *e.g.* spreading or precision placement, is a decision that must be taken directly by the farmer, spectroscopic methods can assist in determining both time and rate. Generally, instruments for fast determination of plant nutritional status can be either hand-held or tractor mounted. Advantages of tractor mounting include the fact that a complete field or area will be measured and the possibility of linking directly to tools for fertilizer application, enabling measuring and fertilizer application in one go. Mapping of the measured area is easily provided by connecting to GPS or other mapping systems from either hand-held or tractor mounted equipment; such systems are already implemented in existing devices.

A hand-held instrument will result in a lower number of individual measurements and cannot be linked directly to fertilizer equipment. However, it is optimal to use as a diagnostic tool for monitoring plant nutritional status throughout the growing season as well as to test plants under suspicion of nutrient deficiency. For some nutrients, particularly micronutrients, varying the applied amount throughout a field may be unnecessary, and a hand-held instrument thus fully satisfies the need for analysis. Other advantages of hand-held equipment as compared to tractor-mounted include the price, which will be significantly lower. Sampling can be done much more precisely, placing the device *e.g.* exactly on a specific leaf or measuring for longer time, meaning that a broader range of methods can be incorporated into a hand-held device. Farming

in less accessible sites, such as rice terraces or vineyards on steep slopes, can benefit from a small, portable instrument but not from a tractor-mounted. Finally, for plant producers with no access to a tractor, a hand-held instrument could be of great assistance, provided they do have access to fertilizer, and the financial means and education to acquire and use such instrumentation.

During development of methods, it is essential that more emphasis is put on specificity of the method to avoid misinterpretations. A low chlorophyll concentration misinterpreted as N deficiency may *e.g.* decrease, instead of increase, nutrient use efficiency (NUE) (Zillmann et al., 2006). Likewise, assessing the variability of measurements is another important issue. Repeating a measurement on the exact same leaf may yield very different results, as demonstrated for NIR in this thesis. Sampling a high number of leaves may therefore be required to obtain a realistic indication of the nutritional status of a field or part of a field. This is especially important for hand-held instruments where a more limited number of measurements is performed.

References

Barker, M., and Rayens, W. (2003). Partial least squares for discrimination. *Journal of Chemometrics* 17**,** 166-173. doi: 10.1002/cem.785.

Chen, M., Chen, J., and Sun, F. (2008). Agricultural phosphorus flow and its environmental impacts in China. *Science of the Total Environment* 405**,** 140-152. doi: 10.1016/j.scitotenv.2008.06.031.

Cozzolino, D., and Moron, A. (2004). Exploring the use of near infrared reflectance spectroscopy (NIRS) to predict trace minerals in legumes. *Animal Feed Science and Technology* 111**,** 161-173.

Dealdana, B.R.V., Criado, B.G., Ciudad, A.G., and Corona, M.E.P. (1995). Estimation of Mineral-Content in Natural Grasslands by Near-Infrared Reflectance Spectroscopy. *Communications in Soil Science and Plant Analysis* 26**,** 1383-1396. doi: 10.1080/00103629509369379.

Force-A (2013). *Multiplex* [Online]. http://www.force-a.eu/an/multiplex.html. [Accessed 130110 2013].

Force, L., Critchley, C., and Van Rensen, J.J.S. (2003). New fluorescence parameters for monitoring photosynthesis in plants - 1. The effect of illumination on the fluorescence parameters of the JIP-test. *Photosynthesis Research* 78**,** 17-33. doi: 10.1023/a:1026012116709.

Gao, C., Sun, B., and Zhang, T.L. (2006). Sustainable nutrient management in Chinese agriculture: Challenges and perspective. *Pedosphere* 16**,** 253-263. doi: 10.1016/s1002-0160(06)60051-9.

Geladi, P., and Kowalski, B.R. (1986). Partial least-squares regression: a tutorial. *Analytica chimica acta* 185**,** 1-17.

Geladi, P., Macdougall, D., and Martens, H. (1985). Linearization and Scatter-Correction for near-Infrared Reflectance Spectra of Meat. *Applied Spectroscopy* 39**,** 491-500. doi: Doi 10.1366/0003702854248656.

Gilbert, N. (2009). Environment: The disappearing nutrient. *Nature* 461**,** 716-718. doi: 10.1038/461716a.

Harris, D.C. (2007). *Quantitative Chemical Analysis.* USA: WH Freeman.

Hebbern, C.A., Pedas, P., Schjoerring, J.K., Knudsen, L., and Husted, S. (2005). Genotypic differences in manganese efficiency: field experiments with winter barley (Hordeum vulgare L.). *Plant and Soil* 272**,** 233-244. doi: 10.1007/s11104-004-5048-9.

Hofer, A. (1998). Variance component estimation in animal breeding: a review. *Journal of Animal Breeding and Genetics* 115**,** 247-265.

Jiang, H.X., Tang, N., Zheng, J.G., Li, Y., and Chen, L.S. (2009). Phosphorus alleviates aluminum-induced inhibition of growth and photosynthesis in Citrus grandis seedlings. *Physiologia plantarum* 137**,** 298-311.

Kjeldahl, K., and Bro, R. (2010). Some common misunderstandings in chemometrics. *Journal of Chemometrics* 24**,** 558-564.

Liao, H., Wu, J., Chen, W., Guo, W., and Shi, C. (2012). Rapid Diagnosis of Nutrient Elements in Fingered Citron Leaf Using Near Infrared Reflectance Spectroscopy. *Journal of Plant Nutrition* 35**,** 1725-1734. doi: 10.1080/01904167.2012.698352.

Lin, Z.-H., Chen, L.-S., Chen, R.-B., Zhang, F.-Z., Jiang, H.-X., and Tang, N. (2009). CO2 assimilation, ribulose-1, 5-bisphosphate carboxylase/oxygenase, carbohydrates and photosynthetic electron transport probed by the JIP-test, of tea leaves in response to phosphorus supply. *BMC plant biology* 9**,** 43.

Mason, S., Mcneill, A., Mclaughlin, M.J., and Zhang, H. (2010). Prediction of wheat response to an application of phosphorus under field conditions using diffusive gradients in thin-films (DGT) and extraction methods. *Plant and Soil* 337**,** 243-258. doi: 10.1007/s11104-010-0521-0.

Mclaughlin, N.B., Hiba, A., Wall, G.J., and King, D.J. (2000). Comparison of energy inputs for inorganic fertilizer and manure based corn production. *Canadian Agricultural Engineering* 42**,** 9-17.

Ntech (2013). *GreenSeeker* [Online]. http://www.ntechindustries.com/greenseeker-RT200.html. [Accessed 130110 2013].

Nutrinostica (2013). *NN-easy55* [Online]. http://nutrinostica.dk/Products2.aspx?pageId=8. [Accessed 130110 2013].

Næs, T., Isaksson, T., Fearn, T., and Davies, T. (2004). *Multivariate Calibration and Classification.* West Sussex, UK: NIR publications.

Osborne, B.G., Fearn, T., and Hindle, P.H. (1993). *Practical NIR spectroscopy with applications in food and beverage analysis.* New York: Longman scientific and technical.

Parry, M.a.J., and Hawkesford, M.J. (2010). Food security: increasing yield and improving resource use efficiency. *Proceedings of the Nutrition Society* 69**,** 592-600. doi: 10.1017/s0029665110003836.

Pavia, D.L., Lampman, G.M., and Kriz, G.S. (2001). *Introduction to spectroscopy.* Harcourt College Publishers.

Raven, P.H., Evert, R.F., and Eichhorn, S.E. (1999). *Biology of plants.* New York, USA: W.H. Freeman.

Ripley, B.S., Redfern, S.P., and Dames, J. (2004). Quantification of the photosynthetic performance of phosphorus-deficient Sorghum by means of chlorophyll-a fluorescence kinetics. *South African Journal of Science* 100**,** 615-618.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F.S., Lambin, E.F., Lenton, T.M., Scheffer, M., Folke, C., and Schellnhuber, H.J. (2009). A safe operating space for humanity. *Nature* 461**,** 472-475.

Samborski, S.M., Tremblay, N., and Fallon, E. (2009). Strategies to Make Use of Plant Sensors-Based Diagnostic Information for Nitrogen Recommendations. *Agronomy Journal* 101**,** 800-816. doi: 10.2134/agronj2008.0162Rx.

Schansker, G., Tóth, S.Z., and Strasser, R.J. (2005). Methylviologen and dibromothymoquinone treatments of pea leaves reveal the role of photosystem I in the Chl *a* fluorescence rise OJIP. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1706**,** 250-261.

Schmidt, S.B., Pedas, P., Laursen, K.H., Schjoerring, J.K., and Husted, S. (2013). Latent Manganese Deficiency in Barley can be Diagnosed and Remediated on

the Basis of Chlorophyll *a* Fluorescence Measurements. *Plant and Soil* 372**,** 417-429. doi: 10.1007/s11104-013-1702-4

Schreiber, U., Neubauer, C., and Klughammer, C. (1989). Devices and methods for room-temperature fluorescence analysis. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences* 323**,** 241-251.

Stirbet, A., and Govindjee (2011). On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. *Journal of Photochemistry and Photobiology B-Biology* 104**,** 236-257. doi: 10.1016/j.jphotobiol.2010.12.010.

Swain, S.S., Tripathy, T., Mohapatra, P.K., and Chand, P.K. (2010). Photosynthetic and transpiration responses of in vitro-regenerated Solanum nigrum L. plants to ex vitro adaptation. *In Vitro Cellular & Developmental Biology-Plant* 46**,** 134-141. doi: 10.1007/s11627-009-9269-8.

Tilman, D., Balzer, C., Hill, J., and Befort, B.L. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences* 108**,** 20260-20264. doi: 10.1073/pnas.1116437108.

Wang, F., Sims, J.T., Ma, L., Ma, W., Dou, Z., and Zhang, F. (2011). The Phosphorus Footprint of China's Food Chain: Implications for Food Security, Natural Resource Management, and Environmental Quality. *Journal of Environmental Quality* 40**,** 1081-1089. doi: 10.2134/jeq2010.0444.

Wise, B.M., Gallagher, N.B., Bro, R., Shaver, J.M., Windig, W., and Koch, R.S. (2006). "Chemometrics Tutorial". (Wenatchee, USA: Eigenvector Research Inc.).

Wold, S., Esbensen, K., and Geladi, P. (1987). Principal component analysis. *Chemometrics and intelligent laboratory systems* 2**,** 37-52.

Wold, S., Sjostrom, M., and Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* 58**,** 109-130. doi: 10.1016/S0169-7439(01)00155-1

Yara (2013). *N-sensor* [Online]. http://www.yara.com/products_services/fertilizers/support_services/support_tools/index.aspx. [Accessed 130110 2013].

Zillmann, E., Graeff, S., Link, J., Batchelor, W.D., and Claupein, W. (2006). Assessment of cereal nitrogen requirements derived by optical on-the-go sensors on heterogeneous soils. *Agronomy Journal* 98**,** 682-690. doi: DOI 10.2134/agronj2005.0253.

Paper II

van Maarschalkerweerd, M., Bro, R., Egebo, M. & Husted, S.

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

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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

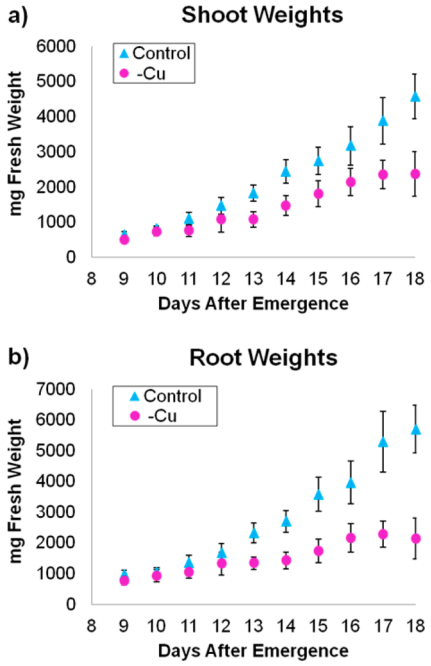

Figure 1. Shoot (a) and root (b) fresh weight of control and increasingly Cu deficient plants from the time series of progressing Cu deficiency. Values are shown as means (n = 24) ± 1 standard deviation (SD). Means of treatments were significantly different in both shoots and roots according to Student's 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 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 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.

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.

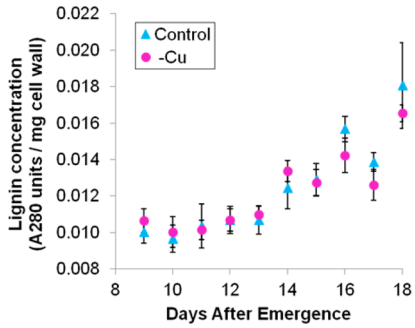

Figure 3. Lignin concentrations in the YFDL of control and increasingly Cu deficient plants from the time series of progressing Cu deficiency. Expressed in units of absorbance at 280 nm per mg isolated cell wall material after derivatization of lignin. Values are shown as means (n = 2) ± 1 SD. Means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

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.

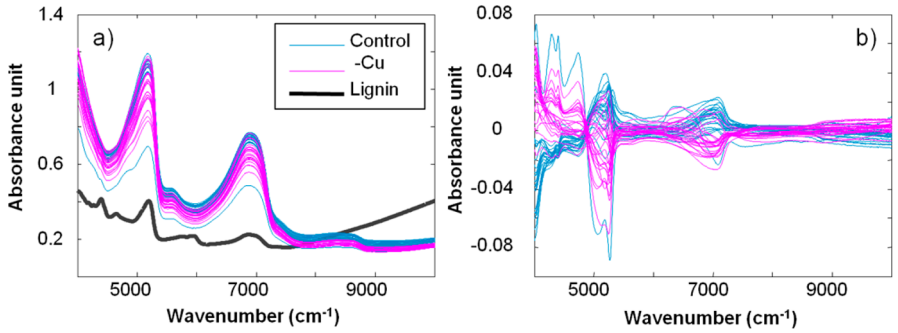

Figure 4. Raw (a) and preprocessed (b) NIR spectra from 13 DAE in the time series of progressing Cu deficiency; the spectrum of pure lignin is inserted in panel a. Preprocessing was carried out using MSC and mean centering.

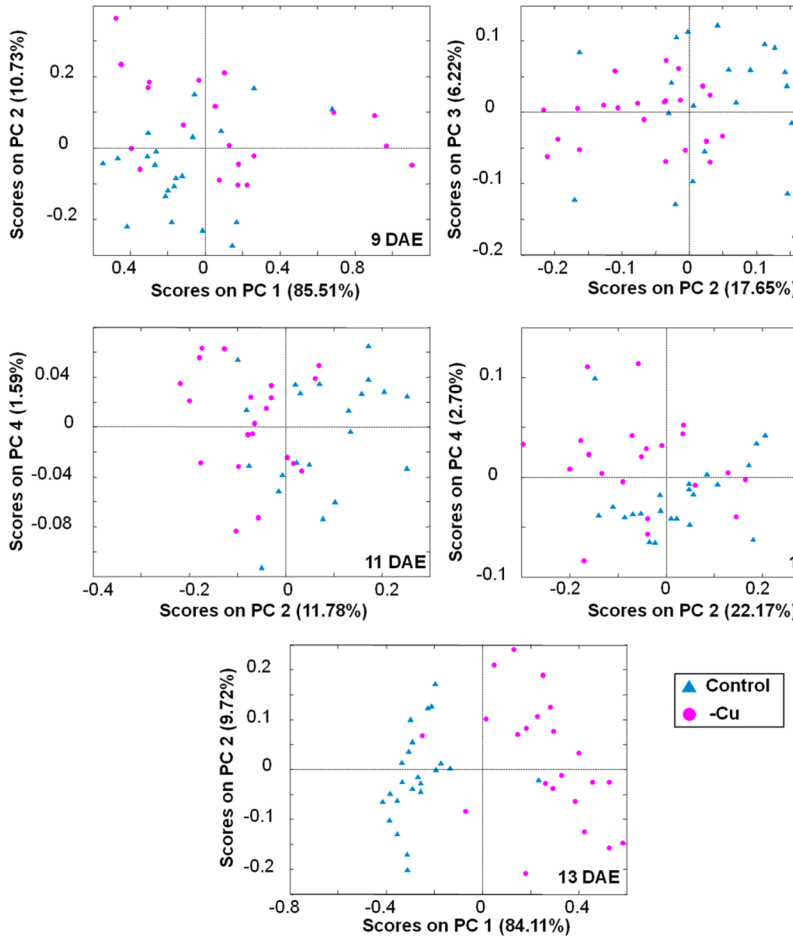

Figure 5. Score plots for PCA models based on NIR spectra from plants in the time series of progressing Cu deficiency. The principal components performing optimal separation of treatments in each model are presented, from 9−13 DAE.

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).

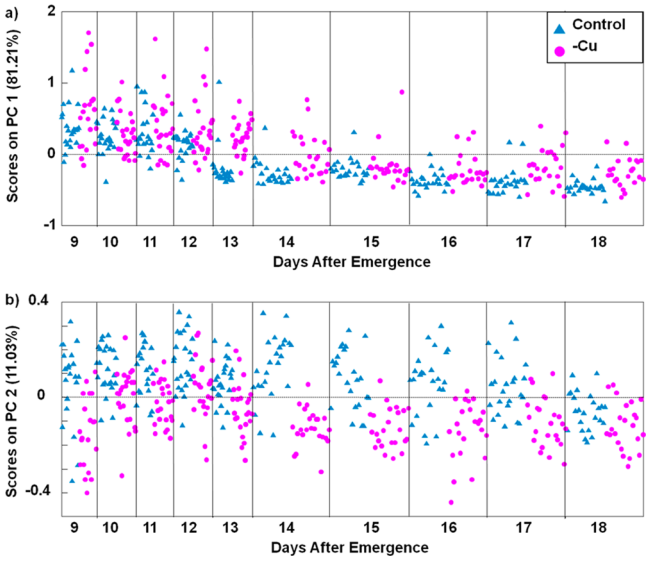

Figure 6. Score plots presenting the first (a) and second (b) principal component versus measuring day (DAE) of a PCA model based on all NIR spectra collected in the time series of progressing Cu deficiency. One batch of control and Cu deficient samples was measured each day. The order of samples within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

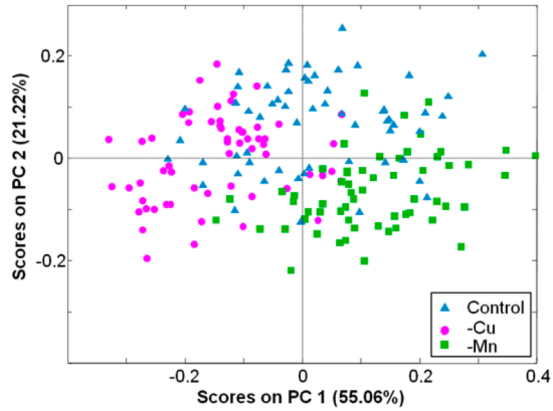

Figure 7. Score plot of a PCA model based on NIR spectra from control, Cu deficient, and Mn deficient plants 31 DAE. The two deficiencies separate mainly along the first principal component, whereas especially control plants tend to have higher values along the second principal component.

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,

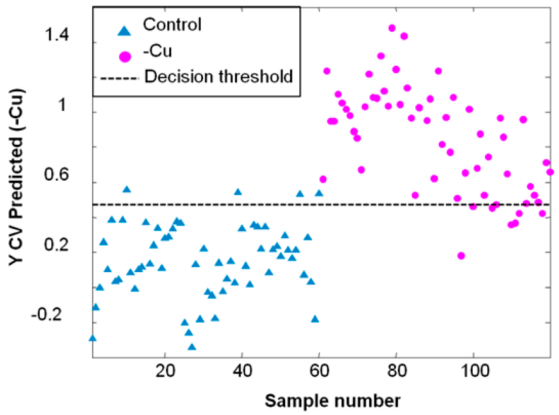

Figure 8. Cross-validated predictions of Cu treatment according to a PLSDA model based on NIR spectra from control and Cu deficient samples measured 32 DAE. Four latent variables were used in the model. The dashed line indicates the optimal separation of treatments, resulting in a clear division.

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

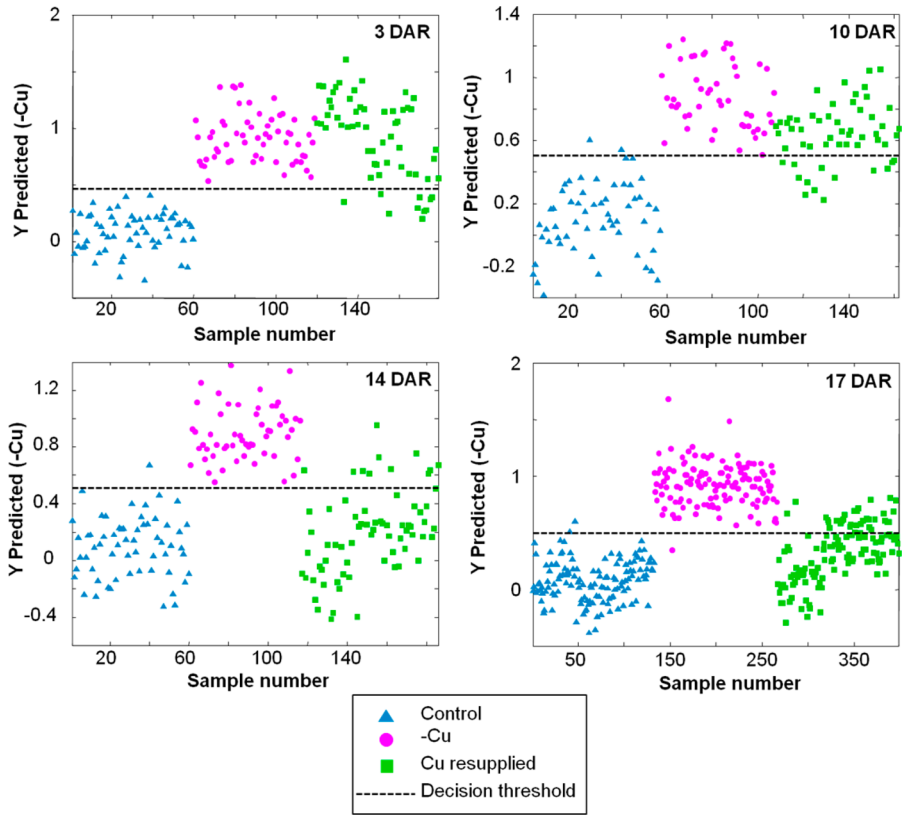

Figure 9. Predictions of Cu resupplied plants according to PLSDA models based on NIR spectra from control and Cu deficient plants. *X*-axes show sample numbers, i.e., they refer to the treatment of the samples. The dashed lines indicate the optimal separation of treatments. Data are derived from 3, 10, 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

REFERENCES

(1) Broadley, M.; Brown, P.; Cakmak, I.; Rengel, Z.; Zhao, F. Function of Nutrients: Micronutrients. In *Mineral Nutrition of Higher Plants*, 3rd ed.; Marschner, P., Ed.; Elsevier: London, 2012; pp 191−248.

(2) Claus, H. Laccases: structure, reactions, distribution. *Micron* **2004**, *35*, 93−6.

(3) Lin, C.-C.; Chen, L.-M.; Liu, Z.-H. Rapid effect of copper on lignin biosynthesis in soybean roots. *Plant Sci.* **2005**, *168*, 855−861.

(4) Solomon, E. I.; Sundaram, U. M.; Machonkin, T. E. Multicopper oxidases and oxygenases. *Chem. Rev.* **1996**, *96*, 2563−2605.

(5) Gou, J. Y.; Park, S.; Yu, X. H.; Miller, L. M.; Liu, C. J. Compositional characterization and imaging of "wall-bound" acylesters of *Populus trichocarpa* reveal differential accumulation of acyl molecules in normal and reactive woods. *Planta* **2008**, *229*, 15−24.

(6) Dell, B. Male-sterility and anther wall structure in copper-deficient plants. *Ann. Bot. (Oxford, U.K.)* **1981**, *48*, 599−608.

(7) Takahashi, M.; Terada, Y.; Nakai, I.; Nakanishi, H.; Yoshimura, E.; Mori, S.; Nishizawa, N. K. Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. *Plant Cell* **2003**, *15*, 1263−1280.

(8) Brennan, R. F.; Bolland, M. D. A. Comparing soil and tissue testing of copper for early growth of wheat. *Commun. Soil Sci. Plant Anal.* **2006**, *37*, 1451−1470.

(9) Rao, N. R.; Ownby, J. D. Development of an ELISA for estimation of the copper nutritional-status of wheat and cotton. *Plant Soil* **1993**, *155*, 453−456.

(10) Chen, M.; Glaz, B.; Gilbert, R. A.; Daroub, S. H.; Barton, F. E.; Wan, Y. Near-infrared reflectance spectroscopy analysis of phosphorus in sugarcane leaves. *Agron. J.* **2002**, *94*, 1324−1331.

(11) Gislum, R.; Micklander, E.; Nielsen, J. P. Quantification of nitrogen concentration in perennial ryegrass and red fescue using near-infrared reflectance spectroscopy (NIRS) and chemometrics. *Field Crops Res.* **2004**, *88*, 269−277.

(12) Graeff, S.; Steffens, D.; Schubert, S. Use of reflectance measurements for the early detection of N, P, Mg, and Fe deficiencies in Zea mays L. *J. Plant Nutr. Soil Sci.* **2001**, *164*, 445−450.

(13) Li, B.; Liew, O. W.; Asundi, A. K. Pre-visual detection of iron and phosphorus deficiency by transformed reflectance spectra. *J. Photochem. Photobiology., B* **2006**, *85*, 131−9.

(14) Wiwart, M.; Fordoński, G.; Żuk-Gołaszewska, K.; Suchowilska, E. Early diagnostics of macronutrient deficiencies in three legume species by color image analysis. *Comput. Electron. Agric.* **2009**, *65*, 125−132.

(15) Spectrum Technologies, Inc. *SPAD 502 Plus Chlorophyll Meter, Product Manual*; Spectrum Technologies Inc.: Fairfax, VA, 2011; http://www.specmeters.com/assets/1/22/2900P_SPAD_502.pdf.

(16) Uddling, J.; Gelang-Alfredsson, J.; Piikki, K.; Pleijel, H. Evaluating the relationship between leaf chlorophyll concentration and SPAD-502 chlorophyll meter readings. *Photosynth. Res.* **2007**, *91*, 37−46.

(17) Cartelat, A.; Cerovic, Z. G.; Goulas, Y.; Meyer, S.; Lelarge, C.; Prioul, J. L.; Barbottin, A.; Jeuffroy, M. H.; Gate, P.; Agati, G.; Moya, I. Optically assessed contents of leaf polyphenolics and chlorophyll as indicators of nitrogen deficiency in wheat (*Triticum aestivum* L.). *Field Crops Res.* **2005**, *91*, 35−49.

(18) Römheld, V. Diagnosis of Deficiency and Toxicity of Nutrients. In *Mineral Nutrition of Higher Plants*, 3rd ed.; Marschner, P., Ed.; Elsevier: London, 2012; pp 299−312.

(19) Zheng, C. F.; Jiang, D.; Liu, F. L.; Dai, T. B.; Jing, Q.; Cao, W. X. Effects of salt and waterlogging stresses and their combination on leaf photosynthesis, chloroplast ATP synthesis, and antioxidant capacity in wheat. *Plant Sci.* **2009**, *176*, 575−582.

(20) Husted, S.; Laursen, K. H.; Hebbern, C. A.; Schmidt, S. B.; Pedas, P.; Haldrup, A.; Jensen, P. E. Manganese deficiency leads to genotype-specific changes in fluorescence induction kinetics and state transitions. *Plant Physiol.* **2009**, *150*, 825−833.

(21) Schmidt, S. B.; Pedas, P.; Laursen, K. H.; Schjoerring, J. K.; Husted, S. Latent manganese deficiency in barley can be diagnosed and remediated on the basis of chlorophyll *a* fluorescence measurements. *Plant and Soil* **2013**, DOI: 10.1007/s11104-013-1702-4.

(22) Lichtenthaler, H. K.; Wellburn, A. R. Determinations of total carotenoids and chlorophylls b of leaf extracts in different solvents. *Biochem. Soc. Trans.* **1983**, *11*, 591−592.

(23) Hatfield, R. D.; Grabber, J.; Ralph, J.; Brei, K. Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: Some cautionary notes. *J. Agric. Food Chem.* **1999**, *47*, 628−632.

(24) Chang, X.; Chandra, R.; Berleth, T.; Beatson, R. Rapid, microscale, acetyl bromide-based method for high-throughput determination of lignin content in Arabidopsis thaliana. *J. Agric. Food Chem.* **2008**, *56*, 6825−6834.

(25) Lu, F. C.; Ralph, J. Reactions of lignin model beta-aryl ethers with acetyl bromide. *Holzforschung* **1996**, *50*, 360−364.

(26) Morrison, I. M. Semi-micro method for determination of lignin and its use in predicting digestibility of forage crops. *J. Sci. Food Agric.* **1972**, *23*, 455−463.

(27) Laursen, K. H.; Hansen, T. H.; Persson, D. P.; Schjoerring, J. K.; Husted, S. Multi-elemental fingerprinting of plant tissue by semi-

quantitative ICP-MS and chemometrics. *J. Anal. At. Spectrom.* **2009**, *24*, 1198−1207.
(28) Laursen, K. H.; Schjoerring, J. K.; Olesen, J. E.; Askegaard, M.; Halekoh, U.; Husted, S. Multielemental fingerprinting as a tool for authentication of organic wheat, barley, faba bean, and potato. *J. Agric. Food Chem.* **2011**, *59*, 4385−4396.
(29) Geladi, P.; Macdougall, D.; Martens, H. Linearization and scatter-correction for near-infrared reflectance spectra of meat. *Appl. Spectrosc.* **1985**, *39*, 491−500.
(30) Martens, H.; Næs, T. *Multivariate Calibration*; John Wiley & Sons: New York, 1989.
(31) Barker, M.; Rayens, W. Partial least squares for discrimination. *J. Chemom.* **2003**, *17*, 166−173.
(32) Osten, D. W. Selection of optimal regression models via cross-validation. *J. Chemom.* **1988**, *2*, 39−48.
(33) Robson, A. D.; Hartley, R. D.; Jarvis, S. C. Effect of copper deficiency on phenolic and other constituents of wheat cell-walls. *New Phytol.* **1981**, *89*, 361−371.
(34) Cogdell, R. J.; Frank, H. A. How carotenoids function in photosynthetic bacteria. *Biochim. Biophys. Acta* **1987**, *895*, 63−79.
(35) Nilsson, T. Comparison of the FOSS NIR Global ANN Calibration against Reference Methods: A Five Year Pan-European Study? In FOSS Analytical A/S: http://www.foss.dk/industry-solution/grain-milling-and-oils/papers; 2011.
(36) Coates, D. B. "Is near infrared spectroscopy only as good as the laboratory reference values?" An empirical approach. *Spectrosc. Eur.* **2002**, *14*, 24−26.
(37) Xu, B.; Wu, Z. S.; Lin, Z. Z.; Sui, C. L.; Shi, X. Y.; Qiao, Y. J. NIR analysis for batch process of ethanol precipitation coupled with a new calibration model updating strategy. *Anal. Chim. Acta* **2012**, *720*, 22−28.
(38) Halgerson, J. L.; Sheaffer, C. C.; Martin, N. P.; Peterson, P. R.; Weston, S. J. Near-infrared reflectance spectroscopy prediction of leaf and mineral concentrations in alfalfa. *Agron. J.* **2004**, *96*, 344−351.
(39) Osborne, B. G.; Fearn, T.; Hindle, P. H. *Practical NIR Spectroscopy with Applications in Food and Beverage Analysis*, 2nd ed.; Longman Scientific and Technical: New York, 1993.
(40) Menesatti, P.; Antonucci, F.; Pallottino, F.; Roccuzzo, G.; Allegra, M.; Stagno, F.; Intrigliolo, F. Estimation of plant nutritional status by Vis−NIR spectrophotometric analysis on orange leaves [*Citrus sinensis* (L) Osbeck cv Tarocco]. *Biosyst. Eng.* **2010**, *105*, 448−454.
(41) Gherardi, M. J.; Dell, B.; Huang, L. Functional copper requirement for catechol oxidase activity in plantation Eucalyptus species. *Plant Soil* **1999**, *210*, 75−81.
(42) Vogel, J. G.; Jokela, E. J. Micronutrient limitations in two managed southern pine stands planted on Florida spodosols. *Soil Sci. Soc. Am. J.* **2011**, *75*, 1117.
(43) Garcia, A. L.; Gallego, J.; Fuentes, V.; Nicolas, N.; Madrid, R. Mineral nutrition of Prunus rootstocks: Leaf concentrations and diagnosis by vector analysis. *Hortscience* **2005**, *40*, 1670−1674.

Paper III

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[*]*Contributed equally to the work*

Using Chlorophyll *a* Fluorescence to Determine Latent Phosphorus Deficiency in Plants

Manuscript in preparation

Using Chlorophyll *a* Fluorescence to Determine Latent Phosphorus Deficiency in Plants

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Abstract

Chlorophyll *a* fluorescence from dark-adapted leaves reflects the functionality of the photosynthetic metabolism of plants. It is expressed in OJIP transients, and here we demonstrate for the first time that the shape of the transients gradually changes during P deficiency, when the so-called I-step disappears. This effect is used successfully to determine whether barley plants (*Hordeum vulgare*, L.) are deficient or non-deficient of P, and to quantitatively assess the P concentrations of deficient plants. The method is shown to be specific for P deficiency. It is furthermore demonstrated that the same effect occurs in tomato plants (*Solanum lycopersicum*, L.), indicating that a general correlation between plant P nutrition and photosynthesis has been identified.

This discovery opens the possibility of producing small, easy to use analytical instruments that will allow for accurate in-situ control of P fertilizer addition to ensure optimal yields without excessive use of the worlds limited P reserves.

Introduction

The world population is estimated to reach 9 billion people by 2050. This means that global agriculture faces the enormous challenge of increasing food production by 70-100%, while at the same time handling the uncertain consequences of global climate change and reducing its environmental footprint[1-3]. A major challenge related to this is the supply and use of P to support plant production[4-6].

Phosphorus is an essential plant nutrient, meaning that plants cannot fulfill a complete lifecycle without P. An estimated 30% to more than 50% of agricultural soils in the world are P deficient and need fertilizer addition to ensure optimal yields[5,6]. However, phosphate rock, the main source of P for fertilizers, is a finite natural resource, and the known, clean rock phosphate reserves are estimated to last as little as 50 years in the gloomiest forecasts[5,7]. This makes P a potential strategic material similar to oil, as very few countries control the vast majority of the known reserves[5-8]. Concurrent with the dwindling of the raw material, an immense over-use is found in some parts of the world, causing eutrophication of water resources while elsewhere, P depletion results in severe yield limitations[5,6]. Phosphorus therefore plays a key role in enabling an increase in food production while excessive use is a significant contributor to the environmental impact of agriculture.

To manage agricultural input optimally, methods for monitoring plant P status during the growing season and testing for acute P deficiency are needed. At present, total P concentrations of plants can be determined, but this requires modern laboratory facilities and involves considerable time consumption for sample transportation, processing and analysis. Here, we present a method that allows for fast, easy, non-destructive, on-site assessment of the plant P status by recording the so-called OJIP transient of a dark-adapted leaf.

When a leaf is hit by actinic light, a large part of it is absorbed and used for photosynthesis. The remaining light is either reflected directly or, for 2-10% of the absorbed light, it is re-emitted as fluorescence from the chlorophyll in the leaves[9-11]. This is commonly known as chlorophyll *a* fluorescence and is closely related to the photosynthesis in the leaf. When a dark-adapted leaf is exposed to continuous, actinic light, the resulting fluorescence forms a so-called Kautsky curve[10], with the rising part typically referred to as the OJIP transient after the four plateaus it forms (Fig. 1). After reaching a maximum, the P-step, at approximately 0.3 seconds, the fluorescence intensity declines again until it reaches a steady state in a matter of minutes[9,10,12].

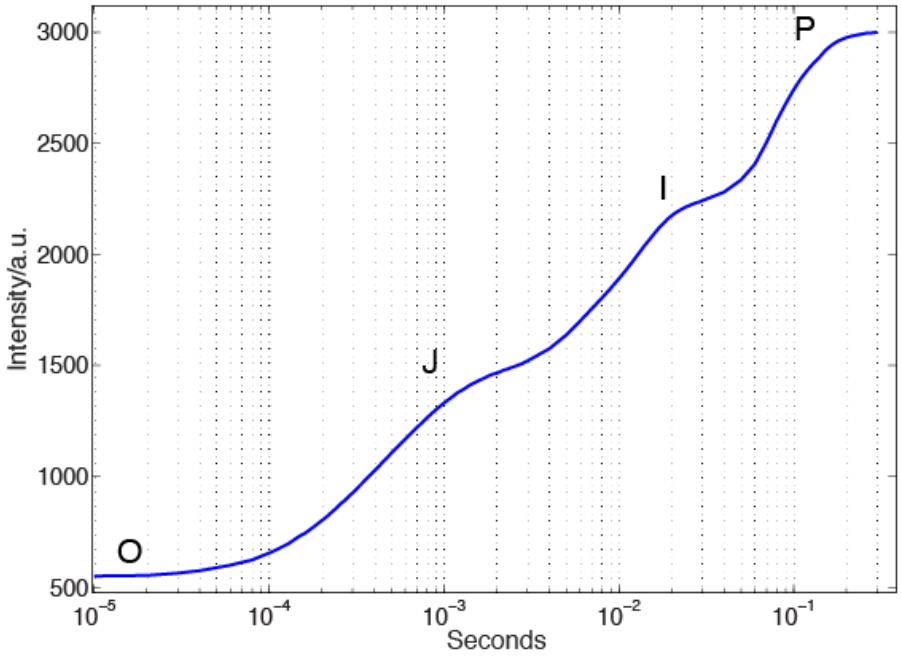


Figure 1: OJIP transient of a healthy leaf plotted on a logarithmic timescale. The features that give rise to the O-J-I-P designation are highlighted. Leaves were dark-adapted for 20 minutes prior to measuring the OJIP transient.

Physiologically, the OJIP transient describes the changes occurring in a leaf that initially has had its photosynthesis halted through dark adaption. This renders the reaction centers of photosystem II (PSII) oxidized, meaning that they are ready to process photons collected by chlorophyll. As the leaf is illuminated with actinic light, the OJIP transient represents the process towards all reaction centers eventually being reduced at peak fluorescence intensity[11,13]. Thus, while the fluorescence itself represents photons that are not processed by the photosynthesis, it does provide a unique insight into the workings of the

photosynthesis, arguably the most important biochemical pathway on the surface of the Earth[11,14,15].

It is not fully understood which processes in the photosynthetic electron transport chain the different steps of the OJIP transient are related to. The first rise (2ms) from O to J is referred to as the photochemical phase due to its dependence on the intensity of the incoming light. Commonly this phase is assumed to reflect the first reduction of plastoquinone A, Q_A, in PSII[11]. The second part, from J over I to P, is called the thermal phase due to its temperature sensitivity. This phase is much slower than the first, and ends when all Q_A is reduced[11]. The physiological reasoning behind the course of the OJIP transient, and the thermal phase in particular, is much debated. J, I and P steps represent kinetic bottlenecks in the electron transport chain, and relations to reduction of plastoquinone and activity in PSI have been suggested for the J-I and I-P phases, respectively[16,17]. However, a wide array of alternative explanations has been presented, and there are strong indications that the view on the mechanisms behind OJIP transients needs to be broadened[18].

Consistent with their known influence on the photosynthetic metabolism, deficiencies of Fe, Cu, Mg, Mn and S have previously been shown to affect the OJIP transient[19-24]. It has also been reported that P has an effect on OJIP transients, although some of the reported effects seem mutually exclusive[25-28]. However, except for Mn[19], no specific effect of deficiency of any one element on the course of the OJIP transients has been described.

We present the unique finding that P deficiency causes the I-step in the OJIP transient to 'straighten' and disappear as a step. It is demonstrated that the effect is specific for P deficiency for both barley and tomato plants and undisturbed by other nutrient deficiencies. Furthermore, it is shown that it is possible to determine whether a plant is P sufficient or deficient, and to quantitatively determine the P concentration in case of deficiency.

Results

Two independent experiments using hydroponically cultivated barley were conducted to test the ability to classify plants as P deficient or sufficient and quantitatively determine the P concentrations. One experiment was conducted in growth chambers at two different climatic conditions and one in greenhouse conditions; thus, the experiments can be considered mutually independent and were used for training- and validation purposes respectively.

Plant Growth and Development

Decreasing levels of plant available P was manifested in lower total biomasses and reduced tillering. Root growth was increasingly prioritized relative to shoot growth as P deficiency became more severe, and high light/low temperature further enhanced this effect (Fig. 2 and Supplementary Table 1). Only plants exposed to a combination of the lowest P levels and the high light/low temperature treatment developed the characteristic, red color of P deficient plants. This anthocyanosis was, however, only detected on the leaf margins of the most P deficient plants (Supplementary Table 1). Carotenoid concentra-

tions were stable for all treatments, whereas chlorophyll concentrations decreased slightly with decreasing P levels (Supplementary Table 1).

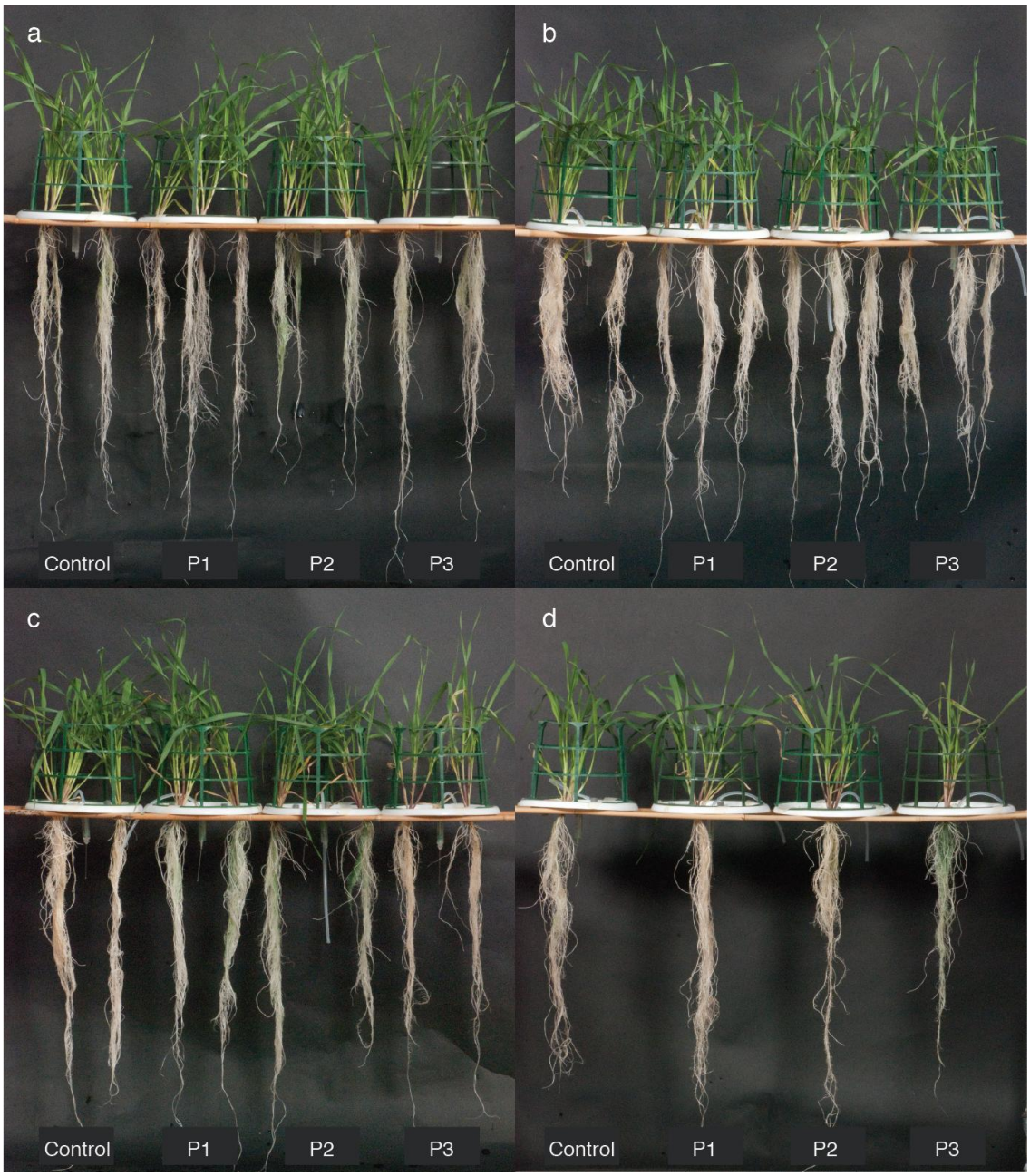


Figure 2: Pictures of plants from four measuring days: 21 (a), 23 (b), 28 (c) and 30 (d) DAT. Increasing levels of P deficiency were induced in P1-P3 plants up to 21 DAT, after which they were completely deprived of P up to 28 DAT. At 28 DAT, P1-3 plants were re-supplied with P at control level. The increased root/shoot ratio of P deficient plants can be noted, but no visual leaf symptoms of P deficiency occurred.

Elemental Concentrations

Phosphorus concentrations in leaves were significantly affected by P treatments as well as climatic conditions, and plants ranging from having a so-called luxury uptake to severe P-deficiency[29] were obtained. Leaf P concentrations were clearly affected by reduced P availability 21 days after transplanting (DAT), and all P deficient plants experienced fur-

ther decreases in leaf P concentrations between 21 DAT and 28 DAT where they were completely deprived of P. Control plants generally had the highest P concentrations, except for plants that were resupplied with P after being heavily deficient. Resupplying resulted in significant increases in P concentrations for plants that were strongly deficient only two days prior (Supplementary Table 2). Concentrations of other essential plant elements remained fairly constant.

OJIP Transients

As P is a highly phloem mobile nutrient, any effect of P deficiency on the OJIP transients is expected to be found in older leaves first. It is easy to distinguish the OJIP transient of the youngest fully developed leaf (YFDL) from the second youngest fully developed leaf (sYFDL) in a plant exposed to the lowest P level (Fig 3a). The OJIP transient from the sYFDL can be discriminated from the YFDL in that the I-step has almost completely straightened, whereas the shapes of O, J and P steps appear unaffected. This difference is enhanced (Fig. 3b) by differentiation of the curves. As the plants become increasingly P deficient, the I-step eventually disappears from both the YFDL and sYFDL, and the I-step reappears for both when plants are resupplied with P (shown in Fig. 4 for YFDL).

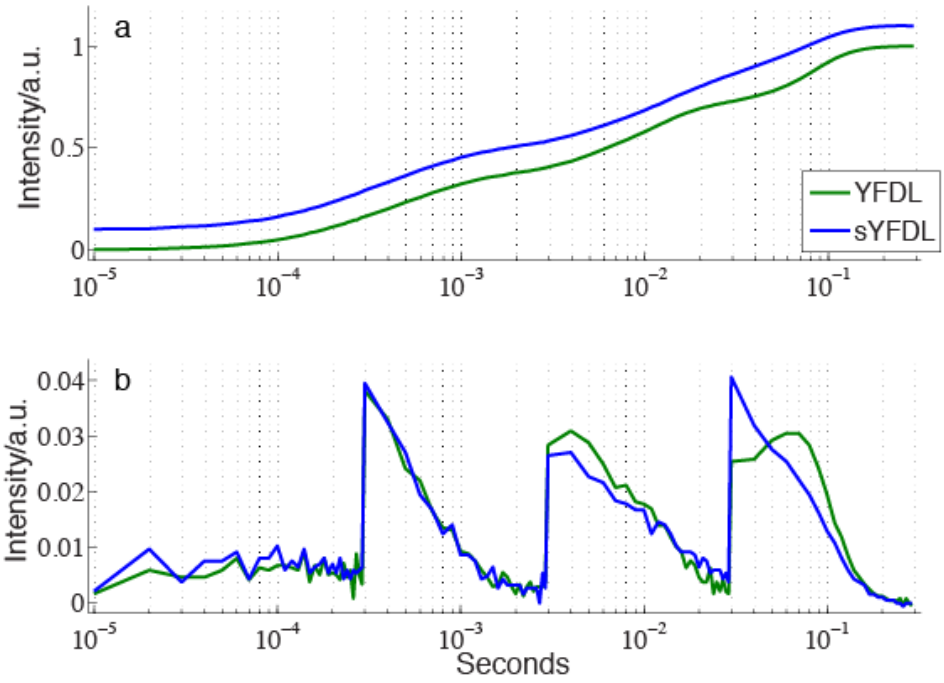


Figure 3: Top panel: OJIP transients of the YFDL and the sYFDL from severely P deficient plant on the first measuring day. Data are normalized, and the transient for the sYFDL has been moved 0.1 units upwards to separate the two OJIP transients. Bottom panel: The same transients after differentiation.

Observing the OJIP transients for the YFDL of a plant exposed to the lowest P level throughout an experiment (Fig. 4) shows the effect of P deficiency. An I-step could be distinguished the first two measuring days (Fig. 4a and 4b), though the plant was increasingly P deficient according to total P concentrations. On the third measuring day (Fig.

4c), the I-step had disappeared. After resupplying plants with P, the I-step was once again clearly recognized two days later (Fig. 4d).

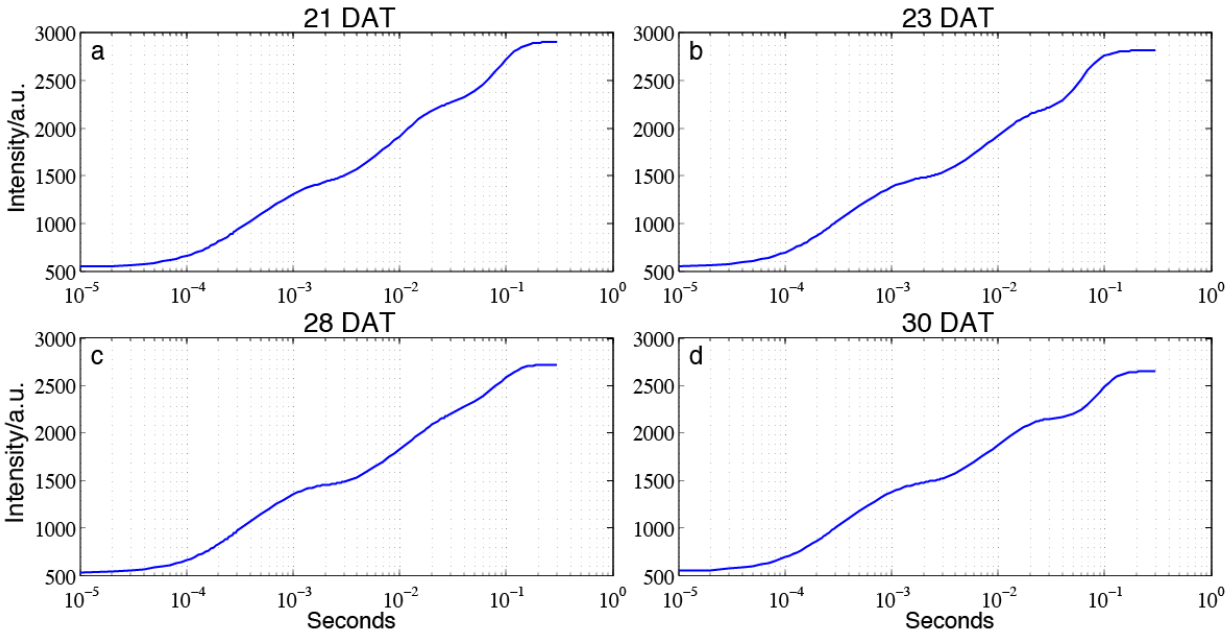


Figure 4: OJIP transients for the YFDL of a plant provided the lowest P level. It is seen that the I-step gradually straightens and almost disappears as the plants become increasingly P deficient from 21 DAT (a) over 23 DAT (b) to 28 DAT (c). The I-step has reappeared (d) two days after plants were resupplied with P at 28 DAT.

Predicting Phosphorus Concentrations

A Partial Least Squares (PLS) regression model was developed using measurements on the YFDL of plants in one experiment (training set) and validated using measurements from the other experiment (validation set). The first 0.3 seconds of the OJIP transients were included, *i.e.* up to the P-step, and all OJIP transients were differentiated to enhance the observed effect of a straightening of the I-step. Reference P concentrations were obtained for the YFDL of individual plants in the validation set, whereas the YFDL's of the five plants in each cultivation unit were pooled for reference analysis in the training set. In five cases, the OJIP transient of one plant deviated strongly from the remaining four plants in the cultivation unit. These outliers were discarded to limit their influence on the PLS model.

The PLS algorithm was clearly unable to model the whole range of P concentrations. However, by only including leaves with P concentrations up to 4000 ppm, it was possible to obtain a PLS model with a low prediction error for P deficient samples, while the majority of leaves with higher concentrations were estimated around the sufficiency threshold of 3000 ppm[29]. Calibration performance and consistency was seen to decrease significantly if higher or lower maximum concentrations were included in the model. Leaves with a P concentration above 4000 ppm are used as an 'internal validation set'. They were not included in the PLS model, but as they derive from the same experiment as the training set, they are not completely independent from this.

A PLS model with three latent variables (LV) was developed using 245 OJIP transients measured on plants from 50 independent cultivation units. All samples had reference P concentrations in the 0-4000 ppm range (Supplementary Fig. 1). This model has a root mean squared error of calibration (RMSEC) of 445 and a root mean squared error of prediction (RMSEP) of 530, calculated for the 24 OJIP transients from the validation set within the 0-4000 ppm range. The regression vector for the PLS model (Supplementary Fig. 2) indicates that predictions are strongly dependent on the straightening of the I-step.

Plotting the predicted P concentrations vs. reference P concentrations for all leaves (Fig. 5), it is clearly noted that the predictions level off. Fitting a 1:1 line, representing a correct PLS prediction, intersecting with a constant line, yields a cut-off value of 2914 ppm (blue curve in Fig. 5). The gradual effect of leveling off is noted in the model predictions, as leaves with a P concentration in the upper part of the model range generally appear to be underestimated (Supplementary Fig. 1).

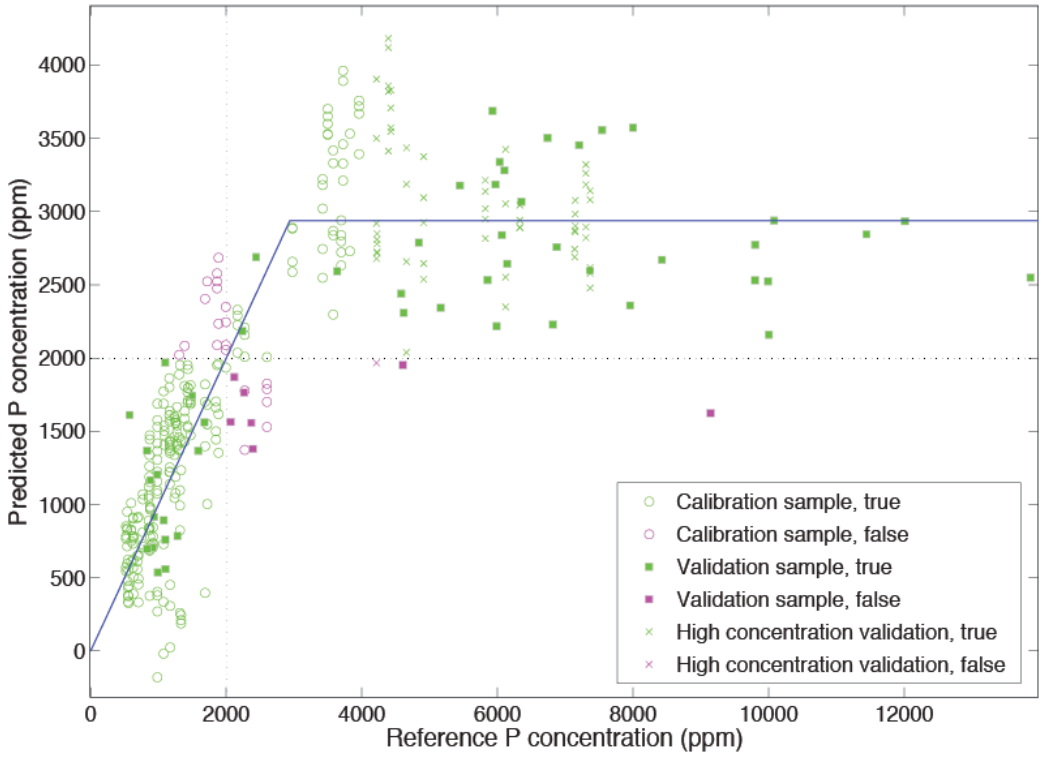


Figure 5: Predicted vs. measured P concentrations for all 360 OJIP transients as predicted by the PLS model. The blue curve represents the optimal fit of a 1:1 line intersecting a constant line at 2914 ppm. The dotted lines indicate the 2000 ppm P sufficiency threshold, and coloring indicates whether or not the PLS model predicts samples correctly according to this threshold.

Setting a P threshold value for the YFDL of 2000 ppm (as indicated in Fig. 5) where plants are consistently considered P deficient[29], only eight leaves from the validation set were falsely classified (Fig. 5). Of these, five were measured to have a P concentration between 2000 and 3000 ppm, thus placing them close to the threshold, and even overlap-

ping with leaves from the training set. In total, 88% of the leaves from the validation set and 98% of the leaves from the internal validation set were predicted correctly according to this threshold. The remaining 12% and 2% of the leaves, respectively, were incorrectly predicted to be deficient.

Validation of Specificity

To test whether the observed effect of P deficiency is specific to P, a Principal Component Analysis (PCA) model was developed for OJIP transients collected in these and previous experiments[19,30,31]. OJIP transients were measured on barley and tomato plants exposed to Ca-, Cu-, Fe-, K-, Mg-, Mn-, N-, P-, S- or Zn-deficiency, along with healthy control plants.

Leaves with a P concentration above 3000 ppm were classified as healthy control leaves, and leaves with a P concentration below 2000 ppm were classified as P deficient. Leaves between these boundaries were discarded for this particular analysis as they may blur the subsequent data visualization. From the previous experiments, OJIP transients were similarly selected to represent plants with a significant deficiency. The first 0.3 seconds of the OJIP transients were included and all OJIP transients were differentiated. In total, the PCA was based on 1029 OJIP transients.

Investigating the scores plot of principal components (PC's) 4 and 5 (Fig. 6), it is evident that the P-deficient samples from all experiments (including both barley and tomato plants) cluster in the first quadrant. Only few samples of other treatments interfere with this cluster. Clusters of Mn, Mg, Cu, S and Fe, which are all nutrients of known importance to the photosynthetic transport chain, are also indicated (Fig. 6); they are seen more clearly in PC's 1-6 (Supplementary Fig. 3). The strongest clustering is found for Mn along PC 1 (Supplementary Fig. 3a), which spans the absolutely major variation in the dataset, 91.92%. PC's 1 and 2 combined show clear clustering of especially S and Cu (Supplementary Fig. 3a). Plotting PC's 2-4 (Supplementary Fig. 3b-c) also indicate clustering of S, along with a less strong clustering of Fe and Mg. PC's 5 and 6 (Supplementary Fig. 3d) present weak clustering of P along with weak clustering of Mg and Mn. No clustering is observed for higher principal components.

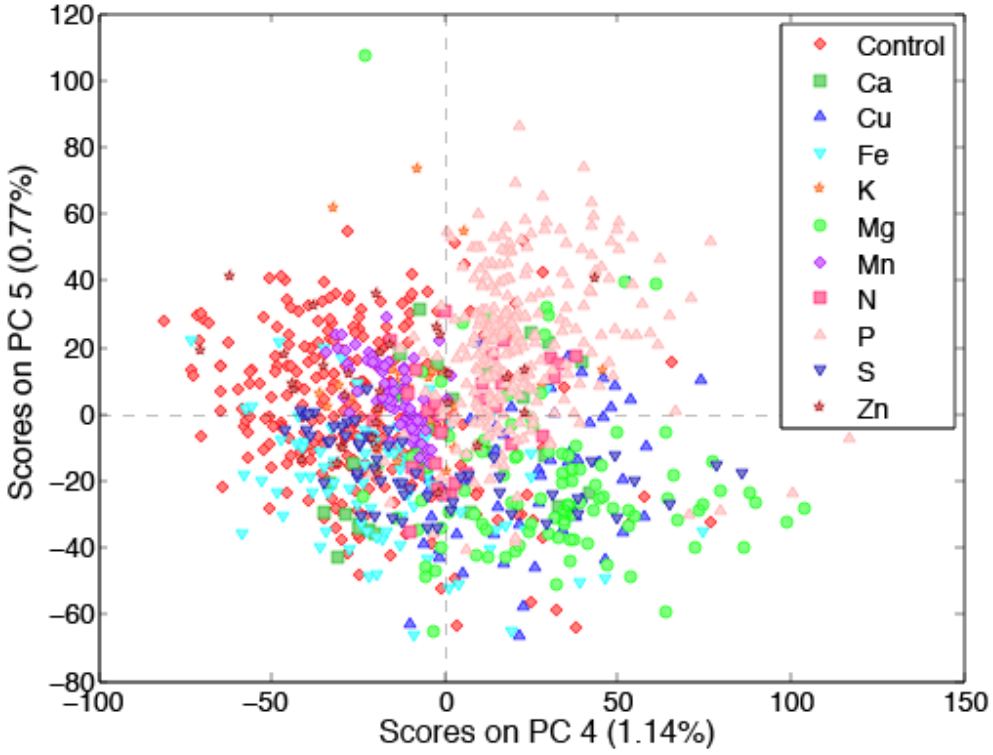


Figure 6: Scores plot showing PC's 4 and 5 of a PCA of OJIP transients from samples with various nutrient deficiencies. Principal Components 4 and 5 explain 1.14% and 0.77% of the total variance respectively. P deficient samples of both barley and tomato plants are seen to cluster in the first quadrant.

Discussion

No visual symptoms of P-deficiency occurred in individual leaves of any of the P-deprived plants used in this study. However, typical signs of latent P deficiency were found, including increased root/shoot ratio, decreased tillering, reduced biomasses and a decrease in chlorophyll concentrations[32]. Carotenoid concentrations were not significantly affected and ICP-OES analyses of the leaves showed that the plants suffered no additional nutrient deficiencies other than P[29]. Only minimal anthocyanin production was observed.

In OJIP transients, a straightening of the I-step was observed when plants suffered from P deficiency (Figs. 3 and 4). As P has no known, direct association with the photosynthetic electron transport chain[32,33], this effect on the shape of the OJIP transient was surprising. It was demonstrated that the effect is reversible when resupplying plants with P (Fig. 4), indicating that it is apparent before the photosynthetic apparatus has been permanently damaged. Consistent with P being a phloem mobile nutrient[32], it was noted that the effect occurred in the sYFDL before the YFDL (Fig. 3); further indicating a direct connection to the plant P nutritional status. The unsupervised clustering of P deficient plants (both tomato and barley) in the PCA scores plot (Fig. 6) furthermore indicates that a unique fingerprint of P deficiency, applying to both mono- and dicotyledonous species, has been identified. The observed effect is, thus, likely to be associated with photosynthesis as such, and can in that case be used to probe the P status of higher plants in general.

To test the ability of OJIP transients to be used for predicting plant P status, a PLS model was successfully generated. As indicated by the fitted cut-off value (Fig. 5), it was possible to predict the P concentration of the YFDL up to a value close to the sufficiency level of barley at 3000 ppm[29]. Leaves with higher P concentrations were all predicted around this concentration (Fig. 5). It was furthermore possible to correctly classify whether leaves contained above or below 2000 ppm for 93% of all validation samples. In fact, only three out of 93 validation samples were falsely predicted to contain below 2000 ppm while having a true P concentration above 3000 ppm, and no validation samples were falsely predicted to contain above 2000 ppm P.

Due to the overall simplicity of performing chlorophyll *a* fluorescence measurements, the observed specificity and successful prediction model indicate that OJIP transients can be used as a valuable probe to determine the P status of crops and establish the need for additional P-fertilizer accordingly; thereby ensuring optimal yields while avoiding excessive use. Considering the clustering seen for the elements Fe, Mg, Mn and Cu (Fig. 5 and Supplementary Fig. 3), it is furthermore possible that a common instrument could be developed to determine the nutritional status of a wider array of nutrients affecting the OJIP transients. More studies are however needed to determine the possibility of such a multi-elemental analysis.

Plants well supplied with P are known to store up to 85-95% of total P as a non-metabolic pool in the cell vacuoles[34]. This is believed to explain the inability to predict P concentrations above the 3000 ppm sufficiency level of the YFDL of barley. When sufficiency has been reached, the processes reflected in the OJIP transients are saturated, and thus, providing additional P has no effect. Likewise it is seen that the approximate linear relation between the shape of the OJIP transient and the P concentrations at deficient levels gradually fades when P concentrations approach the sufficiency level and the plant starts storing P in the non-metabolic pool. This approximate agreement of the fitted maximum value with the P sufficiency level combined with the PLS regression vector (Supplementary Fig. 2) showing a strong dependence on the I-step further support the observed relation between P deficiency and the straightening of the OJIP transient around the I-step.

Because of the observed saturation, it is furthermore assumed that this specific model and fitted maximum level is only applicable for the YFDL of barley; other species with different sufficiency thresholds will be expected to exhibit different maximum levels and hence a different relation to the total P concentration. Applying the model based on the YFDL to OJIP transients collected from the sYFDL gave poor results (data not shown), though the leaves derive from the same species. However, chlorophyll concentrations generally increase with leaf age[35,36], while the proportion of total P bound in RNA decreases with leaf age[36]. This may affect chlorophyll fluorescence intensity and cause a difference in P sufficiency threshold between YFDL and sYFDL. It is therefore unlikely that a common model for YFDL and sYFDL would be successful.

The high importance of P in plant energy metabolism and phosphorylation is well-known, but until now, no direct effect of P concentration on the photosynthetic electron transport chain has been reported[32,33]. The present work demonstrates the impact of P deficiency on

the shape of the OJIP transient, in particular a straightening of the I-step. Changes in the I-P phase have been suggested to indicate an effect on the PSI acceptor side[16], but no clear consensus of the underlying processes of the OJIP transient exists[13,17,37]. The relationship between P status and the shape of the OJIP transient therefore remains speculative. However, the specificity and accuracy of a multivariate regression analysis of OJIP transients to determine P deficiency in plants is established in the above. This finding has the potential to enable an optimization of P fertilizer management in modern agriculture, and thus, support continuous yield growth while minimizing the negative environmental impacts of P fertilizer use.

Materials and Methods

Cultivation of Plants

Barley plants (*Hordeum vulgare*, L., cv. 'Quench') were cultivated in hydroponics. Seeds were pre-germinated for eight days in vermiculite in a greenhouse with minimum day/night temperatures at 18/15°C and a 16/8 hours day/night light regime. Seedlings were transferred to black 4L containers with nutrient solution and aerated using steel medical syringes suspended in the solution, which was changed weekly. Each container held ten (experiment 1) or four (experiment 2) plants fitted in a lid. The control nutrient solution contained 200µM KH_2PO_4, 200µM K_2SO_4, 300µM $MgSO_4 \cdot 7\ H_2O$, 100µM NaCl, 300µM $Mg(NO_3)_2 \cdot 6\ H_2O$, 900µM $Ca(NO_3)_2 \cdot 4\ H_2O$, 600µM KNO_3, 50µM Fe(III)-EDTA-Na, 2.0µM H_3BO_3, 0.8µM $Na_2MoO_4 \cdot 2\ H_2O$, 0.7µM $ZnCl_2$, 7.0µM $MnCl_2 \cdot 4\ H_2O$ and 0.8µM $CuSO_4 \cdot 5\ H_2O$, and control plants were provided control nutrient solution throughout the experiments. In all containers, pH was kept constant at 6.0 ± 0.3 using ultrapure HCl, and 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. All stock nutrient solutions were prepared in Milli-Q water (Milli-Q Element, Millipore, MA, USA).

Experiment 1

After transplanting into hydroponics, plants were divided into two groups, which were exposed to different climatic conditions. Group A was in a growth chamber under normal light settings (400 µmol photons $m^{-2}s^{-1}$) at a 16/8 hours day/night light regime and a constant temperature of 20°C during the whole experiment. Initially, group B was grown under similar conditions; however, ten days after transplanting into hydroponics, the light intensity was increased to 750 µmol photons $m^{-2}s^{-1}$, and the temperature decreased to 15°C. Hydroponic containers were randomized frequently in both growth chambers to avoid any systematic effect of position.

In total, 32 hydroponic containers were cultivated, with 16 in each climatic treatment; four of these were control units, and four were units of each of three different P treatments, P1, P2 and P3. The first ten days after transplanting, P1, P2 and P3 plants were all provided P1 treatment, meaning a reduction of the concentration of KH_2PO_4 to 89µM, as an approximation to sufficient P supply while avoiding luxury uptake. Then, P2 and P3 treatments were induced in the ascribed plants. P2 nutrient solution contained 45µM KH_2PO_4 and P3 nutrient solution contained 9µM KH_2PO_4. Twelve days after induction of

P2 and P3 treatments, *i.e.* 22 DAT, P1, P2 and P3 treatments were deprived completely of P for the rest of the experimental period.

Chlorophyll *a* fluorescence, in the form of OJIP transients, was measured 21 and 28 DAT, and sampled plant material was freeze-dried for reference analysis.

Experiment 2

Experiment 2 was carried out entirely in a greenhouse under climatic conditions as stated above. In total, 16 hydroponic containers were cultivated. The first ten days after transplanting, all containers were provided control conditions. Then three P treatments were induced, P1, P2 and P3, each applied to four containers. P1 and P2 treatments were similar to P1 and P2 in experiment 1, whereas P3 was higher, namely 22µM KH_2PO_4. Depending on the amount of KH_2PO_4, KCl was added to ensure a constant K-concentration for all treatments. 21 DAT, P was completely removed from P1, P2 and P3 treatments. At 28 DAT, P was resupplied by providing all containers with control conditions.

Chlorophyll *a* fluorescence, in the form of OJIP transients, was measured 21, 23, 28 and 30 DAT, and sampled plant material was dried in an oven at 50°C for reference analysis.

Historic Data

OJIP transients from previously conducted experiments[19,30,31] were collected to validate the P specificity of the experimental findings. The transients were measured on barley and tomato (*Solanum lycopersicum*, L.), which had been cultivated in hydroponics under greenhouse conditions comparable to those in experiment 2. Based on the time after nutrient deficiencies were induced, OJIP transients were measured at stages where nutrient deficiencies were expected to be significant and in some cases even visual deficiency symptoms were present. No reference data of elemental concentrations was available.

Measurements

Biomasses

Root and shoot fresh weight (FW) biomasses of plants were determined at harvest, before the YFDL was removed for further analyses. In experiment 2, biomasses were only determined 28 and 30 DAT, *i.e.* the 3rd and 4th measuring day.

Chlorophyll* a *Fluorescence

Chlorophyll *a* fluorescence transients, or OJIP transients, were measured using a Handy PEA chlorophyll fluorimeter (Hansatech Instruments, Kings Lynn, Norfolk, England). The midsection of the youngest fully developed leaf (YFDL) or second youngest fully developed leaf (sYFDL) was dark adapted for at least 20 minutes before measuring. A short, non-actinic light flash was used to adjust the gain of the detector. The actual measurement was conducted by illuminating the leaf with continuous, actinic light at a saturating intensity (>3000 µmol $m^{-2}s^{-1}$) for ten seconds. The light source was three red LED's that were optically filtered to a maximum wavelength of 650 nm. The resulting fluorescence transients were recorded using a PIN photodiode and an optical filter to ensure that only the fluorescence signal, *i.e.* wavelengths >650 nm, was recorded.

In experiment 1, OJIP transients were measured for the youngest fully developed leaf (YFDL) of five plants growing in the same cultivation unit each measuring day. 60 OJIP transients from 12 independent cultivation units were measured for each climate treatment on each of two measuring days. In experiment 2, OJIP transients were measured for both the YFDL and the second youngest fully developed leaf (sYFDL) of one plant from each cultivation unit. Here, 16 OJIP transients from independent units were measured on both the YFDL and sYFDL on each of four measuring days.

Determination of Chlorophyll and Carotenoids

In experiment 1, concentrations of chlorophylls and carotenoids were determined in leaf material from the midsection of the YFDL after measuring OJIP transients. Approximately 50 mg of leaf material were weighed exactly and extracted overnight in methanol. Absorbance was subsequently measured in a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, MA, USA) and chlorophyll and carotenoids concentrations were calculated according to the method described in Lichtenthaler and Wellburn[38].

Anthocyanin Determination

In experiment 1, anthocyanin concentrations were determined in leaf material from the midsection of the YFDL, according to the method described by Ticconi, et al.[39], after measuring OJIP transients. Approximately 50 mg of leaf material were weighed exactly and transferred to 15 ml Falcon tubes, where they were powdered after being submerged in liquid nitrogen for 10 seconds. Two ml 100°C extraction buffer (1-propanol : 37% HCl : H2O in ratio 18:1:81) were quickly added. The samples were incubated at 100°C for 3 minutes, then dark incubated and left overnight at 20°C. Next day, the plant material was centrifuged for 20 minutes at 10,000 g, and the supernatant was transferred to 2 ml tubes. A 60 seconds spin was performed and 300 µL supernatant were transferred to a microplate. An EON microplate spectrophotometer (BioTek Instruments, Winooski, USA) measured the absorbance at 535 and 650 nm, and the anthocyanin content was calculated as A = A535 – A650/g FW.

Multi-Elemental Analysis

Leaf concentrations of P, Fe, Mg, Mn, Zn, K, S and Ca were determined using Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES) (Optima 5300DV, PerkinElmer, Waltham, Massachusetts, USA).

Sample digestion – Experiment 1

After obtaining OJIP transients and saving material for pigment analysis, the YFDL of five plants in each container was freeze-dried. Subsequently, samples were ground in zirconium-coated jars containing a zirconium-coated mill ball in a Retsch MM301 ball-mill. Samples obtained from the same cultivation unit were pooled before grinding to obtain a sufficient amount of sample material. Approximately 20 mg of each dry, ground sample were digested in 500 µL of 70% HNO_3 (Plasmapure, SCP Science) and 250 µL 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).

Samples digestion – Experiment 2
After obtaining OJIP transient, the analyzed YFDL and sYFDL were cut off and oven-dried at 50°C for three days until completely dry. Leaves were subsequently crushed, weighed and the entire sample digested in 2500 µL of 70% HNO_3 (Plasmapure, SCP Science) and 1000 µL 15% H_2O_2 (Ultrapure, Riedel de Haën, Sigma-Aldrich) using a single reaction chamber microoven digestion system (Ultrawave, Milstone S.r.l., BG, Italy).

After digestion, all samples were diluted to 10 ml with milli-Q-water (Milli-Q Element, Millipore) and analyzed directly by ICP-OES as described by Laursen, et al.[40] and Hansen, et al.[41]. Data quality was evaluated by including at least five samples of digested, certified reference material (NIST 1515, apple leaf, National Institute of Standards and Technology, Gaithersburg, MD, USA) in each analytical run. Data was processed using the WinLab32 software (version 3.1.0.0107, PerkinElmer).

Chemometric Analysis
Data was analyzed by the chemometric methods Principal Component Analysis (PCA) and Partial Least Squares (PLS) regression, carried out using Matlab® R2013b (Mathworks, Inc., Natick, MA, USA) and PLS_Toolbox 7.5.0 (Eigenvector Research, Inc., Wenatchee, WA, USA). The PCA is an unsupervised method that enables a simple and comprehensive overview of the major variations in a multivariate data set by reducing the number of dimensions with a minor loss of information. Data is presented using the principal components (PC's) as axes, and in most cases the first few axes will contain information about the major variations within samples in the dataset. The PCA is described more thoroughly in *e.g.* Martens and Næs[42].

The PLS analysis is related to the PCA regarding the focus on major variations in data. However, in PLS the new set of axes, latent variables (LV's), are determined to maximize the covariance between X data and a set of reference data, Y. The resulting model can be used for prediction of Y-values using new X-data as input. The method is further described in *e.g.* Wold, et al.[43].

Before analysis, the spectroscopic data was preprocessed using an approximated derivative, calculating the difference between consecutive time points. The time between consecutive data points increased by an order of magnitude at 0.0003s, 0.003s and 0.03s; enhancing differences between points accordingly. This caused the differences between consecutive data points in the latter part to be enhanced compared to the early part of the OJIP transients. Thus, shape features in general and of the I-P phase in particular are emphasized.

Acknowledgements
We thank Sidsel Birkelund Smith and Kristian Holst Laursen for providing us access to the OJIP transients used in the study of method specificity and for advising on the nutritional status of the given plants.

Supplementary information

Supplementary Table 1: Biomasses in fresh weight (FW), root/shoot relationship and number of tillers for plants in experiment 1 and 2 and chlorophyll and carotenoids concentrations for plants in experiment 1. 'A' designates normal light and temperature conditions; 'B' designates high light and low temperature conditions of experiment 1. In experiment 2 plants were grown in greenhouse conditions. The results are average ± one standard deviation; n=12 and n=4 in experiments 1 and 2, respectively.

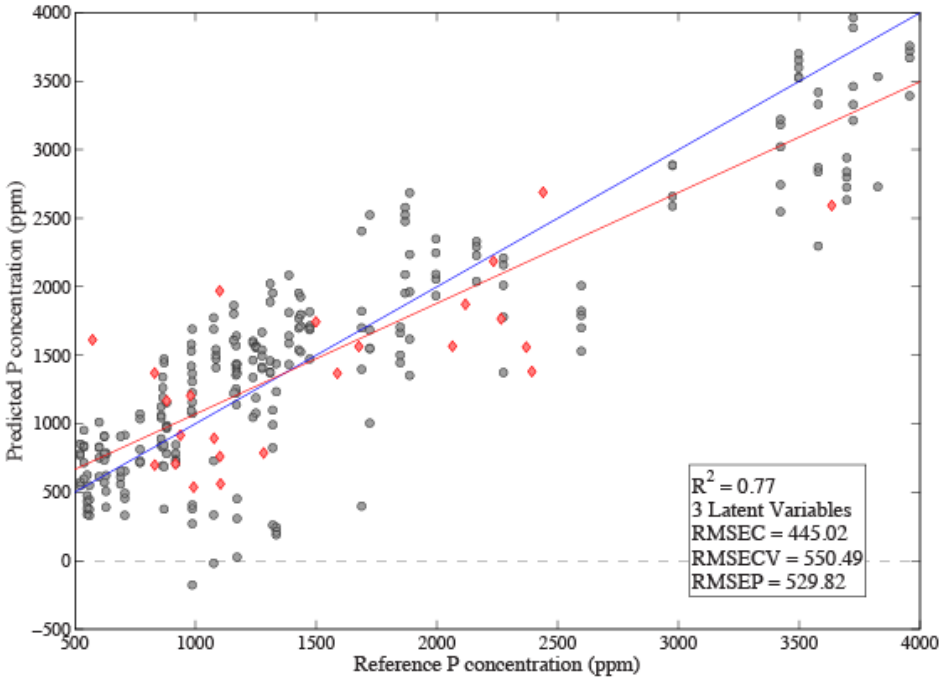
				Shoot (g FW)	**Root (g FW)**	**Root/Shoot**	**Tillers**	**Chlorophyll (mg/g FW)**	**Carotenoids (mg/g FW)**
Experiment 1	21 DAT	A	Control	4.2 ± 0.8	2.2 ± 0.6	0.5	4	2.5 ± 0.1	0.4 ± 0.02
			P1	3.4 ± 0.4	3.0 ± 0.7	0.9	3	2.6 ± 0.1	0.3 ± 0.02
			P2	3.0 ± 0.6	2.7 ± 0.5	0.9	3	2.4 ± 0.2	0.3 ± 0.03
			P3	1.5 ± 0.3	2.4 ± 0.4	1.5	2	2.1 ± 0.2	0.3 ± 0.02
		B	Control	4.1 ± 0.5	3.9 ± 0.8	0.9	5	2.0 ± 0.1	0.3 ± 0.02
			P1	3.6 ± 0.6	3.5 ± 0.9	1.0	4	2.0 ± 0.3	0.3 ± 0.04
			P2	3.0 ± 0.4	3.6 ± 0.8	1.2	3	2.3 ± 0.2	0.4 ± 0.02
			P3	2.1 ± 0.4	3.0 ± 0.6	1.4	3	1.9 ± 0.2	0.4 ± 0.02
	28 DAT	A	Control	9.4 ± 1.4	4.2 ± 1.3	0.5	6	2.6 ± 0.2	0.4 ± 0.04
			P1	6.0 ± 0.9	3.7 ± 0.6	0.6	5	2.7 ± 0.2	0.4 ± 0.02
			P2	4.6 ± 1.0	3.4 ± 0.8	0.7	4	2.4 ± 0.2	0.4 ± 0.03
			P3	2.2 ± 0.5	3.0 ± 0.4	1.4	2	2.0 ± 0.2	0.3 ± 0.02
		B	Control	9.8 ± 2.1	6.8 ± 3.4	0.7	7	2.3 ± 0.2	0.4 ± 0.03
			P1	5.9 ± 1.0	5.0 ± 2.1	0.8	6	2.0 ± 0.2	0.4 ± 0.04
			P2	4.4 ± 0.9	5.4 ± 1.2	1.2	5	1.9 ± 0.1	0.4 ± 0.04
			P3	2.3 ± 0.4	3.2 ± 0.6	1.4	3	1.7 ± 0.1	0.3 ± 0.02
Experiment 2	28 DAT		Control	15.2 ± 2.7	8.5 ± 1.0	0.6			
			P1	10.9 ± 1.1	8.4 ± 1.5	0.7			
			P2	9.8 ± 1.6	8.2 ± 1.3	0.8			
			P3	6.7 ± 0.8	6.2 ± 1.6	1.0			
	30 DAT		Control	20.4 ± 2.7	11.6 ± 2.8	0.6			
			P1	14.0 ± 5.1	10.4 ± 4.2	0.8			
			P2	10.8 ± 1.3	8.1 ± 0.8	0.8			
			P3	6.7 ± 0.8	5.5 ± 1.3	0.8			

Supplementary Table 2: Elemental concentrations as obtained by ICP-OES. 'A' designates normal temperature and light, 'B' designates low temperature and high light. P1-P3 indicate decreasing levels of supplied P. P1-P3 plants were not provided any P between 21 DAT and 28 DAT. In experiment 2, plants were resupplied with P at 28 DAT.

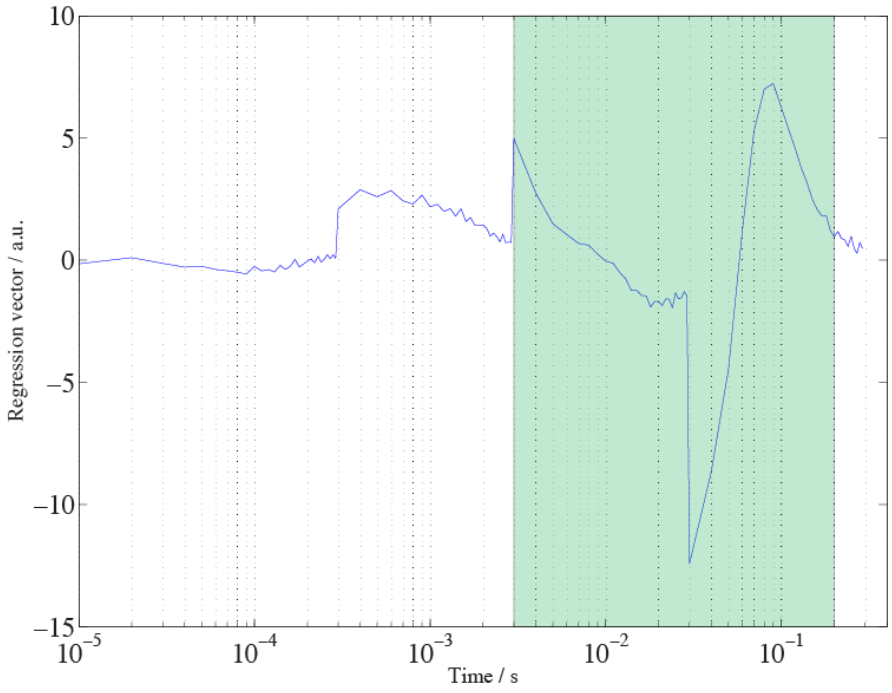
				P (%)		Ca (%)		K (%)		Mg (%)		S (%)	
				YFDL	**sYFDL**	**YFDL**	**sYFDL**	**YFDL**	**sYFDL**	**YFDL**	**sYFDL**	**YFDL**	**sYFDL**
Experiment 1	21 DAT	A	Control	0.72±0.01		0.95±0.12		7.05±0.28		0.40±0.03		0.57±0.03	
			P1	0.37±0.03		1.00±0.04		7.24±0.45		0.41±0.02		0.66±0.01	
			P2	0.20±0.01		0.84±0.04		7.85±0.33		0.34±0.02		0.54±0.02	
			P3	0.12±0.01		0.49±0.02		8.24±0.12		0.22±0.01		0.42±0.02	
		B	Control	0.41±0.04		0.63±0.09		4.24±0.33		0.23±0.02		0.44±0.05	
			P1	0.24±0.05		0.43±0.10		5.12±0.73		0.17±0.03		0.45±0.08	
			P2	0.13±0.01		0.50±0.08		5.83±0.45		0.19±0.03		0.39±0.05	
			P3	0.09±0.01		0.53±0.04		7.71±0.21		0.23±0.03		0.37±0.01	
	28 DAT	A	Control	0.58±0.06		0.78±0.18		5.53±0.66		0.35±0.05		0.61±0.05	
			P1	0.16±0.01		0.75±0.05		6.36±0.36		0.35±0.02		0.52±0.03	
			P2	0.11±0.02		0.61±0.13		7.50±0.20		0.28±0.05		0.44±0.04	
			P3	0.06±0.01		0.63±0.05		8.81±0.38		0.30±0.01		0.38±0.02	
		B	Control	0.41±0.04		0.65±0.21		4.67±0.25		0.20±0.05		0.43±0.1	
			P1	0.11±0.01		0.45±0.11		5.76±0.46		0.16±0.03		0.36±0.05	
			P2	0.09±0.02		0.55±0.05		6.80±0.46		0.18±0.02		0.39±0.02	
			P3	0.06±0.01		0.52±0.03		7.96±0.37		0.24±0.01		0.38±0.03	
Experiment 2	21 DAT		Control	0.62±0.04	0.64±0.11	1.04±0.40	1.07±0.34	8.36±0.45	8.41±0.41	0.46±0.16	0.48±0.11	0.4±0.03	0.4±0.05
			P1	0.54±0.08	0.60±0.04	1.17±0.33	0.83±0.34	8.09±0.57	8.12±0.65	0.51±0.12	0.39±0.14	0.43±0.02	0.41±0.04
			P2	0.21±0.04	0.30±0.08	1.30±0.05	0.67±0.04	7.54±0.40	8.29±0.27	0.58±0.01	0.32±0.02	0.44±0.01	0.43±0.04
			P3	0.12±0.07	0.15±0.02	0.95±0.24	0.78±0.35	6.98±1.62	7.98±0.39	0.44±0.11	0.37±0.15	0.39±0.07	0.42±0.05
	23 DAT		Control	0.70±0.11	0.64±0.06	0.54±0.23	1.17±0.17	8.17±0.32	8.46±0.63	0.28±0.07	0.50±0.07	0.45±0.02	0.42±0.05
			P1	0.44±0.06	0.37±0.01	0.35±0.02	0.93±0.10	7.77±0.18	8.24±0.39	0.21±0.01	0.41±0.02	0.43±0.02	0.42±0.02
			P2	0.24±0.05	0.17±0.10	0.47±0.29	0.74±0.28	7.20±0.91	7.36±0.65	0.23±0.10	0.32±0.10	0.41±0.02	0.41±0.05
			P3	0.12±0.04	0.11±0.07	0.70±0.26	0.94±0.46	7.46±0.24	7.22±1.17	0.32±0.09	0.43±0.18	0.38±0.02	0.38±0.04
	28 DAT		Control	0.65±0.04	0.60±0.06	0.42±0.08	1.06±0.19	6.05±0.63	6.51±0.42	0.26±0.02	0.44±0.07	0.53±0.04	0.48±0.04
			P1	0.17±0.04	0.12±0.02	0.62±0.15	1.19±0.05	7.11±0.76	6.41±0.24	0.29±0.05	0.49±0.02	0.47±0.06	0.39±0.03
			P2	0.11±0.01	0.07±0.01	0.43±0.16	0.94±0.21	6.40±0.20	6.54±0.62	0.21±0.04	0.39±0.08	0.37±0.04	0.35±0.03
			P3	0.09±0.01	0.06±0.00	0.33±0.08	0.77±0.13	6.65±0.33	8.31±0.57	0.18±0.04	0.35±0.05	0.33±0.02	0.33±0.02
	30 DAT		Control	0.60±0.11	0.54±0.10	0.62±0.27	1.19±0.30	7.10±0.24	7.37±0.29	0.32±0.07	0.48±0.14	0.47±0.03	0.43±0.06
			P1	0.89±0.13	0.87±0.12	0.64±0.22	1.15±0.35	7.64±0.52	7.81±0.48	0.29±0.06	0.50±0.14	0.47±0.04	0.41±0.04
			P2	1.03±0.17	0.84±0.09	0.47±0.12	0.98±0.15	8.00±0.23	8.67±0.46	0.23±0.03	0.42±0.05	0.41±0.02	0.38±0.06
			P3	1.09±0.20	0.74±0.13	0.33±0.07	0.72±0.09	7.73±0.27	8.89±1.04	0.19±0.03	0.34±0.05	0.39±0.03	0.36±0.04

Supplementary Table 2 (continued)

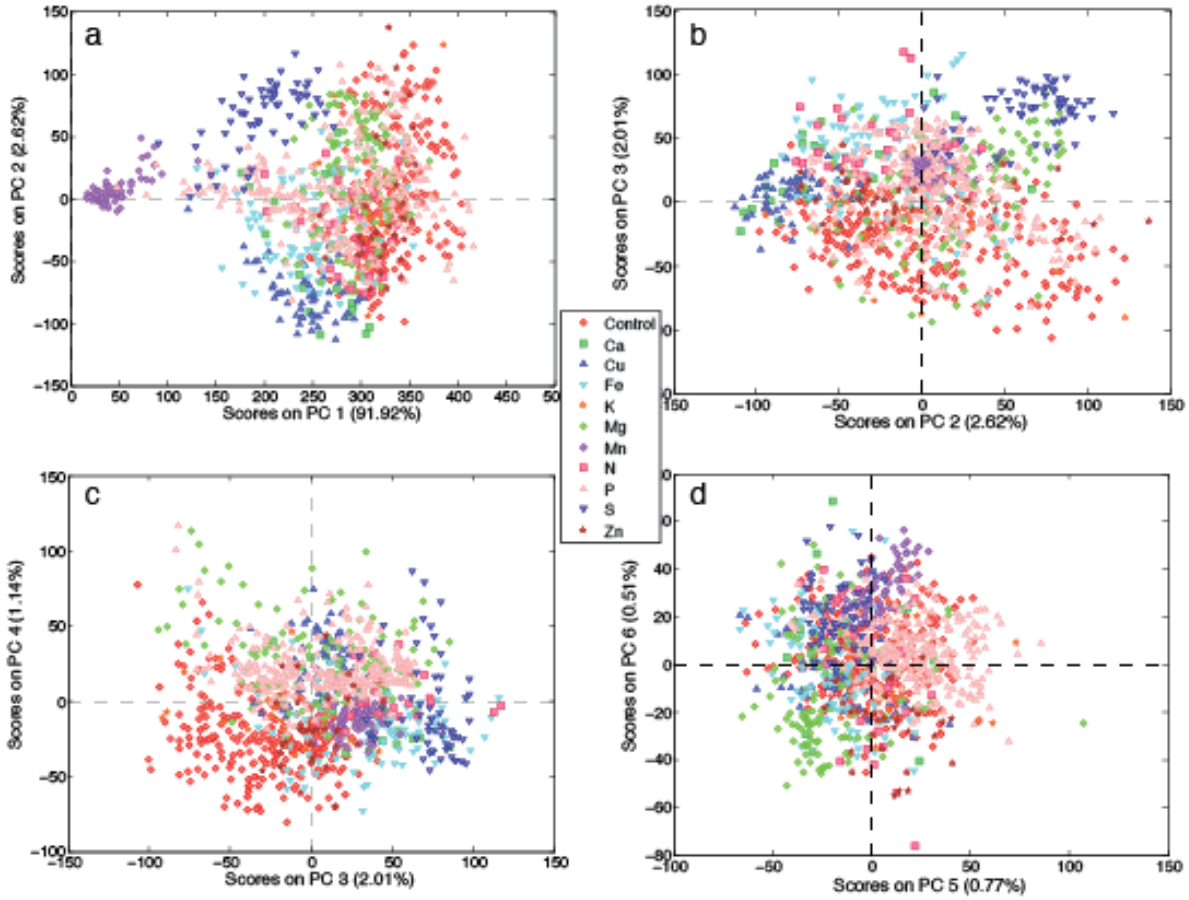
				Fe (ppm)		Mn (ppm)		Zn (ppm)	
				YFDL	**sYFDL**	**YFDL**	**sYFDL**	**YFDL**	**sYFDL**
Experiment 1	21 DAT	A	Control	90 ± 5		70 ± 15		60 ± 5	
			P1	110 ± 15		100 ± 5		80 ± 5	
			P2	100 ± 10		100 ± 15		85 ± 10	
			P3	80 ± 5		100 ± 5		90 ± 5	
		B	Control	60 ± 5		40 ± 10		30 ± 5	
			P1	70 ± 10		35 ± 10		50 ± 10	
			P2	80 ± 5		50 ± 10		60 ± 5	
			P3	100 ± 60		100 ± 10		105 ± 30	
	28 DAT	A	Control	85 ± 5		45 ± 10		45 ± 10	
			P1	85 ± 5		75 ± 10		50 ± 10	
			P2	85 ± 15		80 ± 20		60 ± 10	
			P3	80 ± 5		130 ± 20		90 ± 5	
		B	Control	70 ± 10		30 ± 10		30 ± 5	
			P1	70 ± 20		35 ± 10		40 ± 10	
			P2	70 ± 10		55 ± 5		55 ± 5	
			P3	80 ± 30		100 ± 5		95 ± 10	
Experiment 2	21 DAT		Control	75 ± 5	80 ± 5	160 ± 100	170 ± 85	105 ± 55	115 ± 40
			P1	80 ± 10	85± 15	215 ± 85	125 ± 70	150 ± 50	95 ± 40
			P2	75 ± 10	80± 10	250 ± 25	100 ± 15	150 ± 10	75 ± 10
			P3	60 ± 15	75± 10	190 ± 60	145 ± 90	125 ± 40	115 ± 50
	23 DAT		Control	70 ± 5	65± 10	65 ± 25	160 ± 50	75 ± 10	100 ± 30
			P1	60 ± 5	70 ± 5	50 ± 5	130 ± 10	65 ± 5	85 ± 10
			P2	70 ± 10	60± 10	80 ± 45	120 ± 40	80 ± 10	80 ± 10
			P3	70 ± 10	60± 10	120 ± 30	180 ± 85	100 ± 10	120 ± 30
	28 DAT		Control	70 ± 5	70 ± 5	30 ± 5	90 ± 15	65 ± 25	70 ± 20
			P1	70 ± 10	65 ± 5	90 ± 30	165 ± 20	70 ± 15	80 ± 10
			P2	70 ± 10	60 ± 5	75 ± 30	145 ± 50	65 ± 15	70 ± 15
			P3	60 ± 10	60 ± 5	75 ± 25	130 ± 25	75 ± 10	95 ± 15
	30 DAT		Control	60 ± 5	60 ± 5	50 ± 15	100 ± 40	65 ± 25	75 ± 20
			P1	60 ± 5	60 ± 5	85 ± 35	150 ± 50	70 ± 20	85 ± 25
			P2	60 ± 5	50 ± 5	70 ± 15	145 ± 25	70 ± 10	85 ± 15
			P3	50 ± 10	60± 10	70 ± 15	130 ± 15	80 ± 15	100 ± 25



Supplementary Figure 1: Predicted vs. measured P concentrations based on PLS model with 3 latent variables. Grey circles indicate calibration samples, red diamonds are validation samples.



Supplementary Figure 2: Regression vector for PLS model predicting the P concentration. The high absorbance values of the highlighted area between 0.003s and 0.2s indicate that the more pronounced the I-step is, the higher the predicted P concentration will be.



Supplementary Figure 3: PCA scores plots showing PC's 1-6.

References

1 FAO. Feeding the World, Eradicating Hunger. (United Nations, Food and Agrigultural Organization, ftp://ftp.fao.org/docrep/fao/Meeting/018/k6077e.pdf, 2009).

2 Foley, J. A. *et al.* Solutions for a cultivated planet. *Nature* **478**, 337-342 (2011).

3 Godfray, H. C. J. *et al.* Food security: the challenge of feeding 9 billion people. *science* **327**, 812-818 (2010).

4 Gilbert, N. Environment: The disappearing nutrient. *Nature* **461**, 716-718, doi:10.1038/461716a (2009).

5 Lynch, J. P. Root phenes for enhanced soil exploration and phosphorus acquisition: tools for future crops. *Plant Physiology* **156**, 1041-1049 (2011).

6 MacDonald, G. K., Bennett, E. M., Potter, P. A. & Ramankutty, N. Agronomic phosphorus imbalances across the world's croplands. *Proceedings of the National Academy of Sciences* **108**, 3086-3091 (2011).

7 Edixhoven, J., Gupta, J. & Savenije, H. Recent revisions of phosphate rock reserves and resources: reassuring or misleading? An in-depth literature review of global estimates of phosphate rock reserves and resources. *Earth System Dynamics Discussions* **4**, 1005-1034 (2013).

8 Elser, J. & Bennett, E. A broken biogeochemical cycle. *Nature* **478**, 29-31 (2011).

9 Govindjee, R. in *Chlorophyll a Fluorescence* 1-41 (Springer, 2004).

10 Harbinson, J. & Rosenqvist, E. in *Practical applications of chlorophyll fluorescence in plant biology* (eds Jennifer R DeEll & Peter MA Toivonen) 1-29 (Springer, 2003).

11 Stirbet, A. & Govindjee. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. *Journal of Photochemistry and Photobiology B-Biology* **104**, 236-257, doi:10.1016/j.jphotobiol.2010.12.010 (2011).

12 Govindjee. 63 YEARS SINCE KAUTSKY-CHLOROPHYLL-A FLUORESCENCE. *Australian Journal of Plant Physiology* **22**, 131-160 (1995).

13 Stirbet, A. & Govindjee. Chlorophyll a fluorescence induction: a personal perspective of the thermal phase, the J-I-P rise. *Photosynthesis Research* **113**, 15-61, doi:10.1007/s11120-012-9754-5 (2012).

14 Blankenship, R. E. & Hartman, H. The origin and evolution of oxygenic photosynthesis. *Trends in biochemical sciences* **23**, 94-97 (1998).

15 Lazar, D. Chlorophyll a fluorescence induction. *Bba-Bioenergetics* **1412**, 1-28, doi:10.1016/s0005-2728(99)00047-x (1999).

16 Schansker, G., Tóth, S. Z. & Strasser, R. J. Methylviologen and dibromothymoquinone treatments of pea leaves reveal the role of photosystem I in the Chl *a* fluorescence rise OJIP. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **1706**, 250-261 (2005).

17 Schreiber, U., Neubauer, C. & Klughammer, C. Devices and methods for room-temperature fluorescence analysis. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences* **323**, 241-251 (1989).

18 Schansker, G., Tóth, S. Z., Holzwarth, A. R. & Garab, G. Chlorophyll a fluorescence: beyond the limits of the QA model. *Photosynthesis research*, 1-16 (2013).

19 Husted, S. *et al.* Manganese deficiency leads to genotype-specific changes in fluorescence induction kinetics and state transitions. *Plant Physiology* **150**, 825-833, doi:10.1104/pp.108.134601 (2009).

20 Kastori, R. *et al.* Photosynthesis, chlorophyll fluorescence, and water relations in young sugar beet plants as affected by sulfur supply. *Journal of plant nutrition* **23**, 1037-1049 (2000).
21 Larbi, A., Abadía, A., Morales, F. & Abadía, J. Fe resupply to Fe-deficient sugar beet plants leads to rapid changes in the violaxanthin cycle and other photosynthetic characteristics without significant de novo chlorophyll synthesis. *Photosynthesis Research* **79**, 59-69 (2004).
22 Mallick, N. & Mohn, F. Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga Scenedesmus. *Ecotox Environ Safe* **55**, 64-69 (2003).
23 Tang, N., Li, Y. & Chen, L. S. Magnesium deficiency–induced impairment of photosynthesis in leaves of fruiting Citrus reticulata trees accompanied by up-regulation of antioxidant metabolism to avoid photo-oxidative damage. *Journal of Plant Nutrition and Soil Science* **175**, 784-793 (2012).
24 Yang, G.-H. *et al.* Physiological impacts of magnesium-deficiency in Citrus seedlings: photosynthesis, antioxidant system and carbohydrates. *Trees* **26**, 1237-1250 (2012).
25 Jiang, H. X., Tang, N., Zheng, J. G., Li, Y. & Chen, L. S. Phosphorus alleviates aluminum-induced inhibition of growth and photosynthesis in Citrus grandis seedlings. *Physiol Plantarum* **137**, 298-311 (2009).
26 Lin, Z.-H. *et al.* CO2 assimilation, ribulose-1, 5-bisphosphate carboxylase/oxygenase, carbohydrates and photosynthetic electron transport probed by the JIP-test, of tea leaves in response to phosphorus supply. *BMC plant biology* **9**, 43 (2009).
27 Ripley, B. S., Redfern, S. P. & Dames, J. Quantification of the photosynthetic performance of phosphorus-deficient Sorghum by means of chlorophyll-a fluorescence kinetics. *South African Journal of Science* **100**, 615-618 (2004).
28 Weng, X.-Y., Xu, H.-X., Yang, Y. & Peng, H.-H. Water-water cycle involved in dissipation of excess photon energy in phosphorus deficient rice leaves. *Biol Plantarum* **52**, 307-313 (2008).
29 Reuter, D. J., Robinson, J. B. & Dutkiewicz, C. *Plant analysis: an interpretation manual*. 2 edn, (CSIRO Publishing, 1997).
30 Hebbern, C. A. *et al.* Latent manganese deficiency increases transpiration in barley (Hordeum vulgare). *Physiol Plant* **135**, 307-316, doi:10.1111/j.1399-3054.2008.01188.x (2009).
31 Schmidt, S. B., Pedas, P., Laursen, K. H., Schjoerring, J. K. & Husted, S. Latent Manganese Deficiency in Barley can be Diagnosed and Remediated on the Basis of Chlorophyll *a* Fluorescence Measurements. *Plant and Soil* **372**, 417-429, doi:10.1007/s11104-013-1702-4 (2013).
32 Hawkesford, M. *et al.* in *Mineral Nutrition of Higher Plants* (ed P. Marschner) Ch. 6, 135-189 (Elsevier, 2012).
33 Engels, C., Kirkby, E. & White, P. in *Mineral Nutrition of Higher Plants* (ed P. Marschner) Ch. 5, 85-133 (Elsevier, 2012).
34 Lauer, M. J., Blevins, D. G. & Sierzputowska-Gracz, H. 31P-nuclear magnetic resonance determination of phosphate compartmentation in leaves of reproductive soybeans (Glycine max L.) as affected by phosphate nutrition. *Plant Physiology* **89**, 1331-1336 (1989).
35 Cabello-Pasini, A. & Macías-Carranza, V. Propiedades ópticas de las hojas de vid: reflectancia, transmitancia, absorptancia y concentración de clorofila. *Agrociencia* **45**, 943-957 (2011).

36 Suzuki, Y., Makino, A. & Mae, T. An efficient method for extraction of RNA from rice leaves at different ages using benzyl chloride. *Journal of experimental Botany* **52**, 1575-1579 (2001).
37 Schansker, G., Toth, S. Z., Kovacs, L., Holzwarth, A. R. & Garab, G. Evidence for a fluorescence yield change driven by a light-induced conformational change within photosystem II during the fast chlorophyll a fluorescence rise. *Bba-Bioenergetics* **1807**, 1032-1043, doi:10.1016/j.bbabio.2011.05.022 (2011).
38 Lichtenthaler, H. K. & Wellburn, A. R. Determinations of total carotenoids and chlorophylls b of leaf extracts in different solvents. *Biochem. Soc. Trans.* **11**, 591-592, doi:10.1042/bst0110591 (1983).
39 Ticconi, C. A., Delatorre, C. A. & Abel, S. Attenuation of phosphate starvation responses by phosphite in arabidopsis. *Plant Physiology* **127**, 963-972, doi:10.1104/pp.127.3.963 (2001).
40 Laursen, K. H. *et al.* Multielemental Fingerprinting as a Tool for Authentication of Organic Wheat, Barley, Faba Bean, and Potato. *Journal of Agricultural and Food Chemistry* **59**, 4385-4396, doi:10.1021/jf104928r (2011).
41 Hansen, T. H. *et al.* Micro-scaled high-throughput digestion of plant tissue samples for multi-elemental analysis. *Plant methods* **5**, doi:10.1186/1746-4811-5-12 (2009).
42 Martens, H. & Næs, T. *Multivariate Calibration*. (John Wiley & Sons, 1989).
43 Wold, S., Sjostrom, M. & Eriksson, L. PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* **58**, 109-130, doi:10.1016/S0169-7439(01)00155-1 (2001).

Paper IV

Method and Device for Determining a Nutritional State of a Plant

Submitted September 25, 2013

Patent application

METHOD AND DEVICE FOR DETERMINING A NUTRITIONAL STATE OF A PLANT

The present invention relates in one aspect to a method of determining a nutritional state of a plant. According to a further aspect, the present invention relates to an instrument for determining a nutritional state of a plant.

BACKGROUND OF THE INVENTION

Photosynthesis is a physiological process that is fundamental for the functioning of a plant. A measure of the photosynthesis performance of a plant is therefore a valuable source of information for determining the physiological state of the plant. Photosynthesis converts absorbed light energy into chemical energy that can be used by the plant, and is performed by complex processes in which chlorophyll plays an essential role. While a large part of the light energy absorbed by the plant goes to photosynthesis, some of it undergoes non-photochemical quenching (for a large part through heat dissipation) or is re-emitted by the chlorophyll as fluorescence. Since the three mechanisms – photosynthesis, non-photochemical quenching and chlorophyll fluorescence – are supplied by the same energy source, a variation in performance of one of these processes due to biotic or abiotic factors will be reflected by a variation in at least one of the other processes. However, measuring the complete energy balance for these processes is not practically possible. Nevertheless, the fluorescence signal contains a wealth of information. However, it is a very challenging task to disentangle this information and derive information from fluorescence studies about the physiological state of a plant, which is specific with respect to the influence of a particular parameter, such as deficiency of a particular nutrient or group of nutrients.

The photosynthesis process is subject to a particular kinetics which is reflected in the time dependence of the chlorophyll *a* fluorescence. Upon excitation with actinic light, the chlorophyll *a* fluorescence increases on a time-scale of milliseconds up to about a second from a background fluorescence intensity F_0 up to a maximum fluorescence intensity F_m, and subsequently rolls off on a time-scale of minutes. This phenomenon is called fluorescence induction and can be observed most clearly for leaves that have been dark-adapted initially so as to allow for the largest possible

increase in photosynthesis performance. The rising portion of the fluorescence induction curve exhibits a series of plateaus, commonly denoted by the letters O-J-I-P, and may thus be referred to as the OJIP-rise. Due to the link between chlorophyll *a* fluorescence transients and the photosynthesis process, analysing these transients, and the OJIP-rise in particular, can be used to provide a benchmark test on the overall performance of a given plant. However, it is generally very difficult to isolate the specific influence of a particular parameter on the fluorescence induction transient of a given plant and derive any specific information on the state of the plant, e.g. with respect to a specific nutrient. Exceptionally, it has proven possible to directly link the ratio of the difference between maximum and minimum intensity (F_v) and the maximum intensity (F_m) to the nutritional status of manganese – due to the very direct role manganese plays in the photosynthesis (Husted et al., Plant Physiology, 2009, pp. 825-833). Other approaches suggest a detailed theoretical analysis of the physical mechanisms involved in the photosynthesis processes to retrieve information on the overall health of the plant from an analysis of the fluorescence induction transients (Stirbet et al., Journal of Photochemistry and Photobiology B: Biology, 2011, pp. 236-257). While justified in scientific studies targeted towards understanding the details of the photosynthesis processes, such physical models are not as yet available for implementation in an instrument targeted to determining the nutrient specific nutritional state of a plant. Apart from the above-mentioned method regarding manganese, available methods are generally not suited for distinguishing at least to some level the influences of different nutrients at an early stage of development of the plant, in the field, and with results on the spot. Such information would be desirable, e.g. for facilitating micro-management of fertilization to improve agricultural crops while avoiding excess usage. If available at an early stage, such information would allow for corrective measures before crop damage is permanent.

Therefore, there is a need for a method of determining the nutritional state of a plant with respect to one or more nutrients, which addresses or overcomes at least some of the above-mentioned challenges. The method should at least to some degree be capable of distinguishing between influences of different nutrients. Preferably, the method should be suited for implementation in a mobile measurement instrument suited for field use.

SUMMARY OF THE INVENTION

A first aspect of the invention relates to a method of determining a nutritional state of a plant with respect to one or more nutrients, the method comprising the steps of:

- recording a time series of a fluorescence induction signal of a tissue sample of the plant using a fluorometer device to obtain signal data, wherein the time series at least comprises signal data within the rising portion OJIP of the fluorescence induction signal, and

- determining the nutritional state from an empirical model applied to the signal data, wherein the empirical model is based on pre-recorded reference data and relates reference nutritional states to shape-related features in the time-dependent progression of the fluorescence induction signal.

In the context of the present application, the term 'nutrient' refers to a chemical element that is considered essential for a plant to complete a full life cycle. The term 'nutrient' thus includes the elements oxygen (O), hydrogen (H), carbon (C), nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulphur (S), magnesium (Mg), boron (B), chlorine (Cl), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo) and nickel (Ni).

The nutritional state of a plant with respect to one or more nutrients can be described as healthy, when the plant contains sufficient amounts of these nutrients in order to function properly. If the plant only contains insufficient amounts of one or more nutrients, the lack of nutrient affects the health of the plant, and the nutritional state is described as deficient. The method according to the invention monitors the functioning of the photosynthesis process by recording the fluorescence transient and relating variations in the shape of the fluorescence transient curve to variations in nutrient concentrations in the plant. Consequently, the method detects the ‘bioactive’ part of the nutrient concentration, which is the part that has an effect on the functioning of the plant – in this case the photosynthesis. A plant at any given age is deficient of a nutrient, when the bioactive concentration is insufficient for the plant to

function properly. As such, this method is able to detect deficient nutritional states with respect to one or more nutrients.

Time resolved fluorescence induction data may be obtained using a continuous excitation fluorometer such as the “Handy PEA” commercially available from Hansatech Instruments; or any fluorescence induction measuring device providing a time-resolved fluorescence induction curve with a time-resolution sufficient to resolve the shape of the O-J-I-P features in the rising portion of the fluorescence induction transient. In such an instrument, a tissue sample, such as a leaf, is illuminated by actinic light that activates and saturates photosystem II of the tissue sample. The resulting fluorescence is then collected by a detector capable of measuring the fluorescence intensity emitted by the tissue sample as a function of time. In the “Handy PEA” instrument, the actinic light is provided by an LED-source with a spectral maximum at a wavelength of about 650nm and an intensity of 3000 micro-moles photons/(m^2 * s), and using optical filters to block the LED-source, the chlorophyll *a* fluorescence is collected at wavelengths above 650nm using a PIN-photodiode as detector. The spectral range of detection should include a significant portion of the spectral range of chlorophyll a fluorescence emission in order to provide a reliable representation of induction kinetics. The chlorophyll *a* fluorescence has a pronounced maximum at a wavelength of about 680nm, and a typical range of detection would therefore include this maximum, such as up to 700nm, or even up to 800nm.

The onset of actinic light defines the origin of the time axis, and the fluorescence response from the tissue sample is recorded as a function of time with respect to this origin. Typically, the fluorescence is recorded at intervals that increase with time to account for the logarithmic nature of the fluorescence transient curve exhibiting a fast rise and a slow decline. Typical time intervals between subsequent fluorescent measurements range from 10µs (microseconds) in the beginning to about 10ms (milliseconds) at the maximum of the transient, and may further increase in a logarithmic fashion along the slow decline of the transient.

The method relies on pre-recorded reference data. The reference data is a set of fluorescence transients obtained from plants prepared with a variety of reference

nutritional states with respect to one or more nutrients. The reference states should reflect the range of nutritional states to be tested for. By design, the reference data thus contains the information on how the different nutritional states of the plant affect the fluorescence induction signal. As mentioned above, the chlorophyll *a* fluorescence may be affected by numerous biotic and abiotic factors simultaneously and the wealth of information contained by the fluorescence signal tends to conceal any specific information on the influence of a particular nutrient. The present invention solves this by identifying that differences in shape related features in the time-dependent progression of the signal data are associated to different nutritional states, and utilizing an empirical model to interpret these differences. The term 'shape-related' refers to structures in the progression of the time-dependent fluorescence induction signal; 'shape-related features' are such structures that carry relevant information – here in the form of changes in the shape of the fluorescence induction transient that occur as a function of varying nutritional states. The empirical model is based on the pre-recorded reference data obtained for the various reference nutritional states, and is constructed from the reference data using e.g. multivariate analysis techniques. Based on this empirical model, it is possible to predict the nutritional state of a plant with respect to the nutrient from the signal data.

One of the important merits of the present invention is that shape-related features of the fluorescence induction transient, in particular during the fast OJIP-rise, may be analysed to yield information on the nutritional state of a plant with respect to a particular nutrient or group of nutrients. A further merit of the invention is that, by analysing the shape of the fluorescence induction transient, such information on the nutritional state of a plant may even be independent of genotype and plant species. As a consequence, reference data from one genotype or species may be validated as a reference for signal data of plants of a different one. Yet a further merit of the invention is that an analysis of shape-related features in the fluorescence induction transient yields nutrient-specific information about latent nutrient stress of a plant at a very early stage thus allowing a nutrient-specific treatment of the plant. Early detection of a nutrient deficiency followed by an adequate specific treatment enables correction with a minimum of crop damage, and hence yield loss, as compared to other methods. Yet a further merit of the invention is that a nutrient-specific quantitative analysis of the bioactive level may be achieved.

For some nutrients, the empirical model may allow for a direct and unique identification of the nutritional state with respect to one particular nutrient with a high level of reliability. Examples of such nutrients are phosphorus (P), copper (Cu), manganese (Mn), and sulphur (S). For other nutrients, the empirical model may at least provide a prediction that a plant has a nutrient deficiency, wherein the nutrient may be one of a group of nutrients. Examples of such nutrients are nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), and iron (Fe). Nevertheless, for these other nutrients, the prediction provides information on the nutritional state of the plant, and reduces the ambiguity in identifying the relevant nutrients. The remaining ambiguity may be lifted by providing additional information. Such additional information may be available beforehand, and allow for eliminating some of the other nutrients in that group. Alternatively such additional information may be sought in supplementary analyses.

The method allows for providing a fast, reliable, and accurate test result from a non-destructive/non-invasive optical measurement that may be performed directly on the plant. Preferably, the empirical model is “pre-constructed” and readily available at the time of testing, i.e. when the nutritional state of the plant under test is to be determined. In this case, only the parameters of the empirical model and not the full range of reference data need to be stored for the test. The reference data need only be accessed again when the empirical model needs to be re-validated/re-constructed. This further reduces the need for processing power and data storage capacity, and is therefore particularly advantageous for implementation in mobile devices and for providing a fast test result.

Further according to one embodiment, the method further comprises the step of pre-processing the signal data to enhance non-linear components thereof, wherein the empirical model is constructed from correspondingly pre-processed reference data.

Non-linear features of the time dependent progression of the signal data are enhanced by removing a background of lower order components, thereby enhancing modelling of the nutrient dependent differences between samples in these non-linear features using linear multivariate analysis techniques. Prior to analysis, the

same pre-processing is applied to the time-dependent fluorescence induction transients of the signal data as the one applied to the time-dependent fluorescence induction transients of the reference data when constructing the empirical model. The method relies for the analysis of the shape-related features on the information contained in the non-linear features. Variations in the bioactive levels of nutrients in the plant, and thus changes in the nutritional state of the plant, are detected as variations in the non-linear components of the corresponding fluorescence induction transients. Thereby, a vastly improved analysis of "shape-related features" is achieved.

Further according to one embodiment of the method, pre-processing comprises normalisation, such as multiplicative scatter correction (MSC) or standard normal variate (SNV) transformation, and/or differentiation. Non-linear components in the time-dependent progression of the fluorescence induction signal are enhanced by suppressing lower-order components. For example, this may be achieved by normalization or differentiation with respect to time to suppress linear and sub-linear components. The first derivative removes a constant background. The second derivative removes any linear background. Thereby any non-linear components of the time-dependent progression of the fluorescence induction signal are made more prominent, i.e. enhanced. Similarly MSC and SNV tend to suppress differences in lower-order components between time series. As a consequence, non-linear components become more prominent, i.e. they are enhanced.

Further according to one embodiment of the method, determining the nutritional state comprises classifying the sample in a classification scheme with respect to the one or more nutrients on the basis of the empirical model. Thereby, the method is adapted to provide a classification of the plant under test with regard to its nutritional state as an output. Such output is particularly advantageous in micro-managing agriculture, e.g. in order to detect any unhealthy state, identify the particular nutrients or group of nutrients concerned, and decide on corrective actions to be taken based on that output.

The classification scheme may comprise different levels of detail, e.g. depending on the predictive power of the empirical model with regard to the particular nutrients or

group of nutrients. A classification may be specific for one particular nutrient, and/or comprise a remaining ambiguity by determining a deficiency for at least one out of a group of nutrients. The classification may be performed according to quantitative or semi-quantitative information, such as a number representative of a reliability of the prediction, or classes of deficiency (e.g. slightly/moderately/heavily deficient), wherein the classification scheme may be calibrated against the pre-recorded reference data, e.g. by multivariate analysis techniques.

Further according to one embodiment of the method, determining the nutritional state comprises providing a quantitative prediction representative of a bioactive concentration of the one or more nutrients in the plant on the basis of the empirical model. Thereby, the method is adapted to provide a quantitative output with regard to the nutritional state of the plant under test. The quantitative prediction is calibrated against the pre-recorded reference data obtained on reference tissue samples with a known/measured bioactive concentration of one or more nutrients in the plant. Calibration may be performed using any adequate technique, such as by multivariate analysis techniques. The quantitative output may be consolidated into a value representing the bioactive level of one or more nutrients. A quantitative output is advantageous in order to provide a detailed diagnosis of the plant with regard to its nutritional state. Furthermore, a quantitative output has the advantage that any measures of correction can be adjusted to avoid oversupply of nutrients, and that the uptake of available nutrients by the plant can be monitored.

Further according to one embodiment of the method, the empirical model is constructed from the reference data using a multivariate analysis technique selected from the group of partial least squares regression (PLS), or principal component analysis (PCA). In one variant, the partial least squares regression may be a partial least squares discriminant analysis (PLS-DA).

Further according to one embodiment of the method, the empirical model is constructed by training an artificial neural network using the reference data as a training set. The artificial neural network may be trained to recognise any patterns present in the reference data, which relate changes in the shape-related features to changes in the nutritional state with respect to a particular nutrient or group of nutrients.

Further according to one embodiment, the method further comprises the step of selecting a sub-set of the recorded reference and signal data from one or more time intervals. The method may be optimized with respect to a particular nutrient or a particular group of nutrients by selecting a sub-set of the reference and signal data from one or more time-intervals. The sub-set is selected with regard to shape-related features in the time-dependent fluorescence induction signal that are expected to carry information on the nutritional state of the plant with respect to a particular nutrient or group of nutrients, or with regard to shape-related features that have been identified beforehand to carry such information, e.g. by studying the shape of fluorescence induction transients in the reference data with regard to variations in the nutritional state for this particular nutrient or group of nutrients. Preferably, the selection is made so as to enhance the influence of the shape-related features for this particular nutrient or group of nutrients on the empirical model. Thereby, the empirical model is configured for determining a nutritional state for a particular nutrient or a particular group of nutrients by selecting a sub-set of the data.

One way of identifying shape-related features in the time series of the fluorescence induction curves is recording a set of reference data on tissue samples that have been cultivated to contain varying bioactive concentrations of one or more nutrients (the nutrients of interest), and identifying changes in shape by comparing traces from tissue samples with different bioactive concentrations. Shape-related changes may be enhanced by pre-processing to improve prominence of the features, e.g. differentiation prior to comparison. Sub-sets are then selected from particular time intervals where these shape-related features are prominent.

Further according to one embodiment of the method, the reference and signal data is selected in the range between 10ms and 1s, alternatively between 15ms and 100ms, or between 20ms and 50ms.

According to one advantageous embodiment, the signal data and the corresponding reference data are selected from the time interval between 0s and 10s, covering the full OJIP rise and the peak at P including the beginning of the slow decline.

According to a further advantageous embodiment, the signal data and the corresponding reference data are selected from the time interval between 0s and 3s, covering the full OJIP rise.

According to one preferred embodiment, the signal data, and the corresponding reference data are selected from a time-range covering the so-called 'I-step' in the OJIP rise of the fluorescence induction transient. The I-step is a shape-related feature that comprises information on the nutritional state with respect to a number of nutrients. For example, a pronounced change in shape of this feature is observed for latent phosphorus deficiency. Suitable time-intervals covering the I-step are for example in the range between 10ms and 1s, alternatively between 15ms and 100ms, or between 20ms and 50ms.

For some nutrients, such as phosphorus (P), copper (Cu) and sulphur (S), a high level of reliability of predictions of the nutritional state is observed in particular around the I-step. The information carried by this shape-related feature thus allows for direct and unique determination of the nutritional state with respect to these nutrients – including a quantitative prediction of the bioactive concentration of these nutrients based on the same data.

Further according to one embodiment of the method, the one or more nutrients are selected from the group of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulphur (S), magnesium (Mg), boron (B), manganese (Mn), iron (Fe), zinc (Zn), and copper (Cu).

Further according to one embodiment of the method, the one or more nutrients are selected from the group of phosphorus (P), copper (Cu), manganese (Mn), and sulphur (S). Surprisingly, a high level of reliability for nutrient-specific prediction is observed for these nutrients, thus entailing a low risk of confusion with other nutrients

A second aspect of the invention relates to an instrument configured for performing the method according to any of the above mentioned embodiments. Further embodiments of instruments for determining a nutritional state of a plant with respect to one or more nutrients are cited in the following. The same advantages as mentioned

above with respect to the method for determining a nutritional state are achieved. In particular, the instrument allows for providing a fast, reliable, and accurate test result from a non-destructive/non-invasive optical measurement that may be performed directly on the plant. Furthermore, the instrument may be a mobile device suited for use in the field.

According to a further aspect of the invention, an instrument for determining a nutritional state of a plant with respect to one or more nutrients comprises

- a fluorometer device configured for recording a time series of a fluorescence induction signal of a tissue sample of the plant to obtain signal data, wherein the time series at least comprises signal data within the rising portion of the fluorescence induction signal, and
- an analysis device configured for determining the nutritional state of the plant by applying an empirical model to the signal data, wherein the empirical model is based on pre-recorded reference data and relates reference nutritional states to shape-related features in the time-dependent progression of the fluorescence induction signal.

Further according to one embodiment of the instrument, the analysis device generates an output representative of the nutritional state of the plant. The output may be directly displayed to present data on the nutritional state of the plant under test to a user. The output may be used for deriving a health index for the plant, wherein the health index may be nutrient specific or nutrient-group specific. The instrument may provide the output at an interface for use by a subsequent device, such as a device for diagnosing and/or treating the plant under test. For critical threshold levels, i.e. if a critical state of the plant is determined, the instrument may generate an alarm triggered by the output.

Further according to one embodiment of the instrument, the nutrient specific output is a classification according to a classification scheme based on the empirical model.

Further according to one embodiment of the instrument, the output is a quantitative prediction representative of a bioactive concentration of the one or more nutrients in the plant on the basis of the empirical model.

Further according to one embodiment of the instrument, the one or more nutrients are selected from the group of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulphur (S), magnesium (Mg), boron (B), manganese (Mn), iron (Fe), zinc (Zn), and copper (Cu).

Advantageously according to a further embodiment of the instrument, the one or more nutrients are selected from the group of phosphorus (P), copper (Cu), manganese (Mn), and sulphur (S).

BRIEF DESCRIPTION OF THE DRAWINGS

In the following the invention is further explained by way of examples and with reference to the drawings. The drawings show on

Fig.1 Time series of the fluorescence induction signal recorded from four tissue samples with different phosphorus nutritional states,

Fig.2 The data of Fig.1 after pre-processing by section wise differentiation in each of the time windows A-F to enhance shape-related features,

Fig.3 Median time series of the fluorescence induction signal recorded from a plurality of tissue samples with a specific nutrient deficiency as well as healthy control plants.

Fig.4 The data of Fig.3 after pre-processing by section wise differentiation in each of the time windows A-E to enhance shape-related features,

Fig.5 a PLS prediction of phosphorus concentration (ppm) against independently measured phosphorus concentration (ppm),

Fig.6 a PCA score plot for the first two principal components PC1 and PC2 of a PCA model based on MSC pre-processed data around the I-step.

DETAILED DESCRIPTION / EXAMPLES

Recording of fluorescence induction transients

In all examples, chlorophyll *a* fluorescence transient measurements were performed using the commercially available Hansatech Instrument “Handy PEA”. Using the dark adaption clips fitting this instrument, the tissue samples to be analysed were initially dark adapted for at least 20 minutes, thereby effectively stopping all photosynthesis activity and maximizing the intensity increase of the OJIP-rise. When measuring, the sensor unit of the Handy PEA is placed on the dark adaption clip, thereby allowing the tissue sample to be exposed to the sensor and diodes without letting light in. Initially, a background level was determined by using a short flash of non-actinic light and measuring the response from the tissue sample, and the gain of the detector was adjusted accordingly.

After this initial adjustment, the actual measurement was conducted by illuminating the exposed leaf by three red LED’s that are optically filtered to a maximum wavelength of 650nm. This light is on for the duration of the measurement, and irradiates the tissue sample with a photon flux of at least 3000 µmol m^{-2} s^{-1} so as to effectively saturate the tissue sample with actinic light during the measurement.

Switching on the actinic light induces a chlorophyll *a* fluorescence induction signal with a fast rise of the fluorescence intensity, followed by a slow decline. The fluorescence signal occurs at wavelengths above the wavelength of the actinic light. The intensity of the fluorescence transient was recorded using a PIN photodiode as a detector. Optical filtering is used to ensure that only the longer wavelength (>650nm) fluorescence signal is recorded, thereby avoiding artefacts stemming from actinic light reaching the detector. The PIN-detector integrates the intensity of the induced fluorescence signal over the full spectral range reaching the detector.

A time series of fluorescence intensity measurements was recorded at increasing intervals between successive measurements as a function of total time evolved,

wherein the origin of the total time axis is defined as the point when actinic light is switched on. The following gives an overview of the number of data points recorded for a set of signal data, and intervals between successive data points in different time windows of the total time evolved after switching on the actinic light. The chosen distribution of time intervals is one example of a somewhat logarithmic increase of the time intervals with total time evolved. However, other distributions may easily be conceived by the skilled person in order to adapt the time resolution of the recorded signal data with respect to the overall logarithmic nature of the progression of the fluorescence intensity as a function of total time evolved.

Data point number	Interval / s	Total time / s	Time window
1–30	10×10^{-6}	$(0{,}0–0{,}30) \times 10^{-3}$	A
31–57	$0{,}10 \times 10^{-3}$	$(0{,}40–3{,}0) \times 10^{-3}$	B
58–84	$1{,}0 \times 10^{-3}$	$(4{,}0–30) \times 10^{-3}$	C
85–111	10×10^{-3}	0,04–0,30	D
112–138	0,10	0,40–3,0	E
139–145	1,0	4,0–10	F

For the purpose of the present examples each fluorescence induction data set thus consists of 145 individual measurements, and provides a time-dependent fluorescence curve that is best visualized using a logarithmic time-scale. The data sets cover the first 10 seconds of the fluorescence induction transient including the fast fluorescence rise, the peak intensity, and the beginning of the slow decline. When merely studying the fluorescence rise up to and including the intensity peak, only the first approximately 3 seconds are recorded, of which the first second may suffice.

Reference nutritional states

Two experiments where performed providing two sets of reference nutritional states, each covering a range of physiological states of spring barley (cv. Quench) with different levels of phosphorus deficiency. In both cases, barley plants were grown hydroponically, thereby allowing for a clear control of the nutrient-levels available to each plant. Plants were divided into four different treatments that varied slightly between the two experiments, but in both cases they consisted of one control treatment (P0), and three P-treatments (P1-P3) with decreasing P concentration.

In both experiments, spring barley, cv. Quench, was germinated for eight days in soaked Sorbix vermiculite in a greenhouse with minimum day/night temperatures at 18/15 ^{o}C and 16 hours of light each day (minimum 250-300 µmol photons m^{-2} s^{-1}), and subsequently grown hydroponically in 4 L opaque cultivation units. The nutrient solution in the growing units is based on a standard control treatment with: 200 µM KH_2PO_4, 200 µM K_2SO_4, 300 µM $MgSO_4 \cdot 7H_2O$, 100 µM NaCl, 300 µM $Mg(NO_3)_2 \cdot 6H_2O$, 900 µM $Ca(NO_3)_2 \cdot 4H_2O$, 600 µM KNO_3, 50 µM Fe(III)-EDTA-Na, 0.8 µM $Na_2MoO_4 \cdot 2H_2O$, 1 µM $MnCl_2 \cdot 4H_2O$, 0.7 µM $ZnCl_2$, 0.8 µM $CuSO_4 \cdot 5H_2O$, 2 µM H_3BO_3. To avoid EDTA poisoning, the concentrations of the micronutrients were however halved in the first growth week after transfer to the cultivation units.

Each cultivation unit was continuously aerated with filtered air and the nutrient solution was renewed weekly to ensure optimal nutrient availability of all essential nutrients, except phosphorus for the plants where phosphorus deficiency is induced. pH was kept constant at 6.0±0.3 using ultrapure HCl.

The Control treatment had ample amounts of all nutrients during the whole experiment, and P1, P2, and P3 treatments were supplied with decreasing amounts of P. In both experiments, the intention of the P1 level was to estimate the P level, so that P1 just fulfilled the P requirement of the plants, while avoiding the luxury uptake found in Control plants. Based on previous experience, the concentration of phosphorus in the P1 units was set at 89 µM (i.e. 89 µM of KH_2PO_4). The P2 levels were set at 50% of the P1 level in both experiments, and the P3-level was set at 10 % and 25% of the P1 level in experiment 1 and 2 respectively. Specifically for experiment 2, the potassium removed when reducing the concentration of KH_2PO_4 was replaced by adding additional KCl – thereby keeping a constant level of potassium throughout the experiment across all four treatments.

Experiment 1 – Climate chamber

The germinated plants were transferred to 32 cultivation units, each containing 10 plants, and divided into two groups (A and B) with 16 units each (day 1). Group A was cultivated in a climate chamber under normal light settings (400 µmol photons m^{-2} s^{-1}) and a constant temperature of 20 ^{o}C during the whole experiment. Group B was cultivated in a climate chamber with the same initial settings as A, however,

when the reduced P levels were induced, the settings were changed into high light intensity (750 µmol photons m^{-2} s^{-1}) and a constant temperature at 15 ^{o}C. Twice a week the positions of the units were randomized within each chamber.

The 16 units in each climate chamber were divided into the four different P treatments (Control, P1, P2, and P3). For the first 10 days, P1, P2, and P3 units were all supplied with nutrient solution P1, to avoid a luxury uptake of P in the pre-cultivation phase but allow the production of healthy biomass. After 10 days, the three limited P levels described above were induced. At day 21, all three P-limited treatments were replaced by a nutrient solution containing no phosphorus.

The plants were sampled twice in each climate chamber during the experimental period. The first sampling was conducted at day 21 and the last sampling at day 28 where the P1-3 plants had been completely deprived of phosphorus for seven days. At each sampling, the youngest fully developed leaf from five different plants (which were subsequently harvested) in each cultivation unit were analysed using the Handy PEA, and leaves from each separate cultivation unit were subsequently pooled together as one sample and analysed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). This way, the average phosphorus-content of the youngest fully mature leaf from each cultivation unit is obtained, and this was used as a reference value for each of the five chlorophyll *a* fluorescence measurements on individual plants in that cultivation unit.

Experiment 2 – Greenhouse

The germinated plants were transferred to 16 cultivation units each containing 4 plants (day 1), and were cultivated under the same greenhouse conditions as for the germination period. The 16 cultivation units were divided into the four different phosphorus treatments (Control, P1, P2, and P3), and their positions were randomized twice a week.

All four different treatments were given control-level nutrient supply during the first ten days. On day 10, the P1, P2, and P3 levels were induced using their respective nutrient solutions. On day 21, phosphorus was removed completely from the P1-3

treatments. On day 28, all cultivation units were supplied with control-level nutrient concentrations to observe a potential effect of phosphorus re-supply.

The plants were sampled four times during the experimental period, at day 21, day 23, day 28, and day 30. One plant from each cultivation unit was harvested, and chlorophyll *a* measurements were performed on both the youngest fully developed leaf and the second youngest fully developed leaf. Unlike experiment 1, each leaf was subsequently analysed separately using ICP-OES, thereby giving a specific reference value for each chlorophyll *a* fluorescence measurement.

Data-processing

One of the central merits of the present invention rests in the insight that an appropriate analysis of shape-related features of the progression of the fluorescence induction transient, and in particular of the rising portion, yields nutrient-specific information about the state of deficiency with respect to a specific nutrient. To that end, changes in the shape-related features as compared to the unstressed state with respect to the specific nutrient are detected and analysed. According to the present invention, the nutritional state is determined from these changes by constructing an empirical model, which on the basis of reference data relates specific reference nutritional states to shape-related features in the fluorescence induction transients and applying this empirical model to the signal data obtained from a plant under test. The reference data is pre-recorded fluorescence induction time series obtained from reference plants, i.e. plants that are prepared to be in a particular nutritional state with respect to the specific nutrient as described above. The signal data are fluorescence induction time series obtained from a plant under test. The empirical model is constructed using multivariate analysis techniques.

Pre-processing

Pre-processing was applied to the fluorescence induction data in order to enhance shape-related features. For a given analysis, both signal data and the corresponding reference data used to construct the empirical model were pre-processed using the same pre-processing technique. A number of different pre-processing techniques may be employed. Two of these pre-processing techniques are presented here. In one analysis the same algorithm as commonly used for multiplicative scatter correc-

tion of infrared spectra was applied to a sub-set of time-dependent fluorescence induction data selected from a time-range around the I-step (between 2.6ms and 100ms). The multiplicative scatter correction algorithm turned out to work surprisingly well as pre-processing for the purpose of enhancing shape-related features in the progression of the fluorescence induction data. In a further analysis, numerical differentiation was applied by taking the difference in fluorescence signal of two subsequent data points. The differentiation is done section wise for each of the above-mentioned time windows A-F.

Fig.1 and Fig.2 illustrate the enhancement of the shape-related features by differentiation for different bioactive concentrations of a particular nutrient, here phosphorus. Fig.1 shows the recorded fluorescence induction signal as a function of time for four tissue samples with different phosphorus concentration on a log/linear scale. The data covers the OJIP rise and the beginning of the subsequent decline in the time interval between 0s–10s. Fig.2 shows the corresponding data after pre-processing by section wise differentiation applied to the fluorescence induction signal in each of the time windows A-F. Note for example, the pronounced change in the shape-related feature around the I-step at about 50ms for phosphorus deficient plants (treatments P1-P3) as compared to the healthy state (treatment P0).

Fig.3 and Fig.4 illustrate the enhancement of the shape-related features by differentiation for different nutrient species. Fig.3 shows the fluorescence induction signal as a function of time from tissue samples with a nutrient specific deficiency on a log/linear scale. The data covers the OJIP rise up to and including the peak at ‘P’ in the time interval between 0s–3s. Three of the fluorescence induction traces shown are from a plurality of tissue samples in nutrient deficient states with respect to phosphorus, sulphur, and copper, respectively. The fourth fluorescence induction trace is from healthy tissue samples. Fig.4 shows the corresponding data after pre-processing by section wise differentiation applied to the fluorescence induction signal in each of the time windows A-E. Clear nutrient-specific differences in shape are observed for different tissue samples.

Empirical model

In one analysis, an empirical model is constructed from fluorescence induction transients recorded in the time-range between 0s–10s for the set of reference nutritional states of Experiment 1 and Experiment 2, pre-processed by differentiation. The differentiation is obtained section wise as described above. The empirical model is constructed by Partial Least Squares Regression. A phosphorus concentration of about 3000ppm–4000ppm is the threshold commonly used in practical agriculture for when a plant is considered 'healthy'. A higher phosphorus concentration is thus not 'bioactive' in the plant, and this 'luxury uptake' will instead be stored as a reserve for potential later needs. The empirical model is therefore constructed for all those plants that have an independently measured phosphorus concentration of below 4000ppm. Fig.5 shows a graph of the cross-validated predicted phosphorus concentration (ppm) against measured phosphorus concentration (ppm) for Experiment 1 and Experiment 2. The diagonal line indicates the desired 1:1 relation of perfect prediction. Cross-validation was performed using four randomly selected subsets, and the number of latent variables was chosen to minimize the difference between the root mean squared error of calibration (RMSEC) and the RMSE of cross-validation (RMSECV). Outliers were removed based on Hotelling's T^2 vs. residual plots. The reliability of the prediction is characterized by an R^2-value of about 0.8 indicating a good correlation between measured and predicted phosphorus concentration. Furthermore, the phosphorus concentrations above 3000 ppm (measured) are seen to be slightly under-predicted. This 'cut-off' of the phosphorus concentration predicted on the basis of fluorescence induction data as compared to the total phosphorus concentration in the plant as measured by an independent method, is in agreement with the fact that the fluorescence induction is sensitive to the bioactive fraction of the nutrients contained in the plant rather than the total concentration. As the threshold for barley is between 3000-4000 ppm, it is in full agreement with theory that samples should tend to be underestimated when the phosphorus concentration is above 3000ppm.

In a further analysis, an empirical model is constructed from a collection of fluorescence induction transients recorded for the set of reference nutritional states of Experiment 1 and Experiment 2 with respect to phosphorus and from a library of fluorescence induction transients recorded for deficient nutritional states with respect to

nutrients from the group of phosphorus (P), manganese (Mn), boron (B), nitrogen (N), potassium (K), calcium (Ca), sulphur (S), magnesium (Mg), iron (Fe), zinc (Zn), and copper (Cu). A sub-set of the data in the collection is selected by only taking into account fluorescence induction signals around the I-step, in the time interval between 2.6ms and 100ms. The empirical model is constructed using principal component analysis (PCA). Prior to performing the PCA and constructing the empirical model, the fluorescence induction transients were pre-processed by applying the Multiplicative Scatter Correction (MSC) algorithm, which is commonly used for pre-processing of infrared spectra. Such an algorithm is commercially available, for example in the PLS_toolbox 7.3.1 software by Eigenvector Research for Matlab The algorithm acts surprisingly well to enhance shape-related features of the fluorescence induction transients by suppressing lower order artefacts in the time-dependent fluorescence induction signal. PCA is an unsupervised multivariate analysis method. Any pattern detected in the data can therefore give a reliable indication of the presence of systematic dependencies in the data. Fig.6 shows a PCA score plot for the first two principal components PC1 and PC2, wherein PC1 explains 74.5% of the variance in the above mentioned ensemble of data, and PC2 explains 18.7% in this data. Together, PC1 and PC2 thus explain more than 90% of the variance in the data. The plot shows a large number of individual fluorescence induction transients in the ensemble, each represented by a point with the PC1 and PC2 coefficients as the x- and y-coordinates, respectively. Each point thus represents a particular nutritional state with respect to a particular nutrient. Different symbols represent fluorescence induction transients for different nutrients. A clear clustering of phosphorus deficient states is observed in the lower right portion of the graph (hollow triangles). Furthermore, Sulphur deficient states cluster in a region at the top of the graph (hollow squares), and Copper deficient states appear to agglomerate to the left (hollow circles). This plot underlines the capability of the method according to the invention, at least for some nutrients, to uniquely identify a state of nutritional deficiency, and possibly even quantify the level of deficiency.

In yet a further analysis, a partial least squares discriminant analysis (PLSDA) regression is made on the same library of fluorescence induction transients as used for the PCA above. Unlike above however, the entire of fluorescence induction transients in the time interval from 0s–3s is pre-processed by section-wise differentia-

tion. As PLSDA is a supervised method unlike PCA, the results are cross validated by dividing the data into ten random subsets. After removing outliers a PLSDA model is made using 9 latent variables; giving the confusion matrix shown in Table 2, and the confusion table shown in Table 3. These results further corroborate the ability of this method to provide nutrient specific predictions based on shape-related features in the fluorescence induction transients.

Table 2

Class:	*TP*	*FP*	*TN*	*FN*
Ctrl	0.53	0.14	0.86	0.47
Ca	0.27	0.03	0.97	0.73
Cu	**0.82**	**0.04**	**0.96**	**0.18**
Fe	0.15	0.04	0.96	0.85
K	0.19	0.01	0.99	0.81
Mg	0.13	0.01	0.99	0.87
Mn	**0.76**	**0.01**	**0.98**	**0.24**
N	0.40	0.04	0.96	0.60
P	**0.74**	**0.04**	**0.96**	**0.26**
Zn	0.29	0.04	0.96	0.71
S	**0.75**	**0.10**	**0.90**	**0.25**
B	0.69	0.04	0.96	0.31

Table 2 shows the cross-validated confusion matrix for the PLSDA model (9 latent variables) showing the relative number of true positive (TP), false positive (FP), true negative (TN) and false negative (FN) for each class/nutrient. The nutrients that show promise in terms of specificity are highlighted as bold.

Table 3

Predicted vs. Actual	*Ctrl*	*Ca*	*Cu*	*Fe*	*K*	*Mg*	*Mn*	*N*	*P*	*Zn*	*S*	*B*
Predicted as Ctrl	204	0	7	56	8	54	3	0	25	9	17	0
Predicted as Ca	2	13	6	5	3	14	1	10	7	1	1	0
Predicted as Cu	**26**	**7**	**129**	**10**	**4**	**2**	**4**	**1**	**4**	**2**	**1**	**0**

Predicted vs. Actual	*Ctrl*	*Ca*	*Cu*	*Fe*	*K*	*Mg*	*Mn*	*N*	*P*	*Zn*	*S*	*B*
Predicted as Fe	17	1	7	29	2	9	0	3	10	2	6	0
Predicted as K	5	2	0	1	9	5	0	0	2	1	1	2
Predicted as Mg	9	0	0	4	0	28	0	0	4	0	3	0
Predicted as Mn	**7**	**3**	**0**	**4**	**0**	**3**	**55**	**0**	**6**	**0**	**0**	**0**
Predicted as N	0	9	0	28	2	22	0	19	3	2	1	1
Predicted as P	**9**	**8**	**1**	**9**	**0**	**13**	**5**	**8**	**213**	**4**	**2**	**0**
Predicted as Zn	29	4	1	1	13	8	0	4	7	14	0	2
Predicted as S	**58**	**0**	**7**	**39**	**0**	**48**	**2**	**0**	**1**	**0**	**95**	**0**
Predicted as B	16	1	0	8	7	3	6	2	6	13	0	11

Table 3: Confusion table showing the cross validated most probable predicted classes versus the reference classes. The nutrients that show promise in terms of specificity are highlighted. The PLSDA further supports the capability of the method according to the invention to provide nutrient specific information on the nutritional state of a plant, which at least for some nutrients may even be a unique identification of one or more nutrients related to a deficiency state with a high degree of reliability.

CLAIMS

1. Method of determining a nutritional state of a plant with respect to one or more nutrients, the method comprising the steps of
   - recording a time series of a fluorescence induction signal of a tissue sample of the plant using a fluorometer device to obtain signal data, wherein the time series at least comprises signal data within the rising portion of the fluorescence induction signal, and
   - determining the nutritional state from an empirical model applied to the signal data, wherein the empirical model is based on pre-recorded reference data and relates nutritional states to shape-related features in the time-dependent progression of the fluorescence induction signal.

2. Method according to claim 1, further comprising the step of pre-processing the signal data to enhance non-linear features thereof.

3. Method according to claim 2, wherein pre-processing comprises normalisation and/or differentiation.

4. Method according to any of the preceding claims, wherein determining the nutritional state comprises classifying the sample in a classification scheme with respect to the one or more nutrients on the basis of the empirical model.

5. Method according to any of the preceding claims, wherein determining the nutritional state comprises providing a quantitative prediction representative of a bioactive concentration of the one or more nutrients in the plant on the basis of the empirical model.

6. Method according to claim 4 or claim 5, wherein the empirical model is constructed from the reference data using a multivariate analysis technique selected from the group of partial least squares regression (PLS), or principal component analysis (PCA).

7. Method according to any of the preceding claims, further comprising the step of selecting a sub-set of the recorded reference and signal data from one or more time intervals.

8. Method according to claim 7, wherein the reference and signal data is selected in the range between 10ms and 1s, alternatively between 15ms and 100ms, or between 20ms and 50ms.

9. Method according to any of the preceding claims, wherein the one or more nutrients are selected from the group of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulphur (S), magnesium (Mg), boron (B), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu).

10. Method according to claim 9, wherein the one or more nutrient is selected from the group of phosphorus (P), copper (Cu), manganese (Mn) and sulphur (S).

11. Instrument for determining a nutritional state of a plant with respect to one or more nutrients, the instrument comprising
    - a fluorometer device configured for recording a time series of a fluorescence induction signal of a tissue sample of the plant to obtain signal data, wherein the time series at least comprises signal data within the rising portion of the fluorescence induction signal, and
    - an analysis device configured for determining the nutritional state of the plant by applying an empirical model to the signal data, wherein the empirical model is based on pre-recorded reference data and relates nutritional states to shape-related features in the time-dependent progression of the fluorescence induction signal.

12. Instrument according to claim 11, wherein the analysis device generates an output representative of the nutritional state of the plant.

13. Instrument according to claim 12, wherein the output is a classification according to a classification scheme, wherein the classification is based on the empirical model.

14. Instrument according to claim 12, wherein the output is a quantitative prediction representative of a bioactive concentration of the one or more nutrients in the plant on the basis of the empirical model.

15. Instrument according to any one of claims 12–14, wherein the one or more nutrients are selected from the group of nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), sulphur (S), magnesium (Mg), boron (B), manganese (Mn), iron (Fe), zinc (Zn), and copper (Cu).

ABSTRACT

A method and an instrument for determining a nutritional state of a plant with respect to one or more nutrients is provided. The method comprises the steps of recording a time series of a fluorescence induction signal of a tissue sample of the plant using a fluorometer device to obtain signal data, wherein the time series at least comprises signal data within the rising portion of the fluorescence induction signal, and determining the nutritional state from an empirical model applied to the signal data, wherein the empirical model is based on pre-recorded reference data and relates nutritional states to shape-related features in the progression of the fluorescence induction signal.

Fig. 1

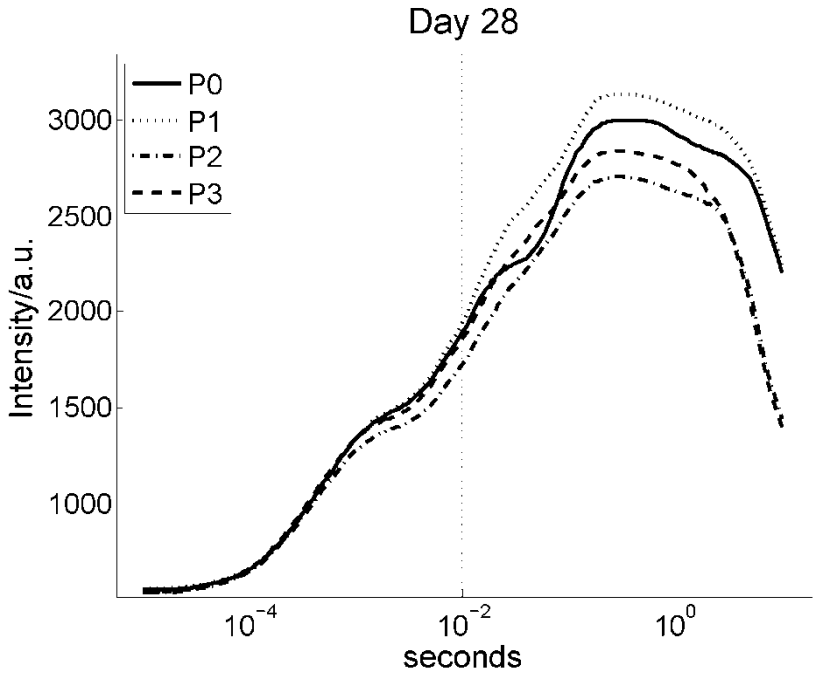

Day 28
P0
P1
P2
P3
3000
2500
2000
1500
1000
Intensity/a.u.
10^{-4}
10^{-2}
10^{0}
seconds

Fig.1

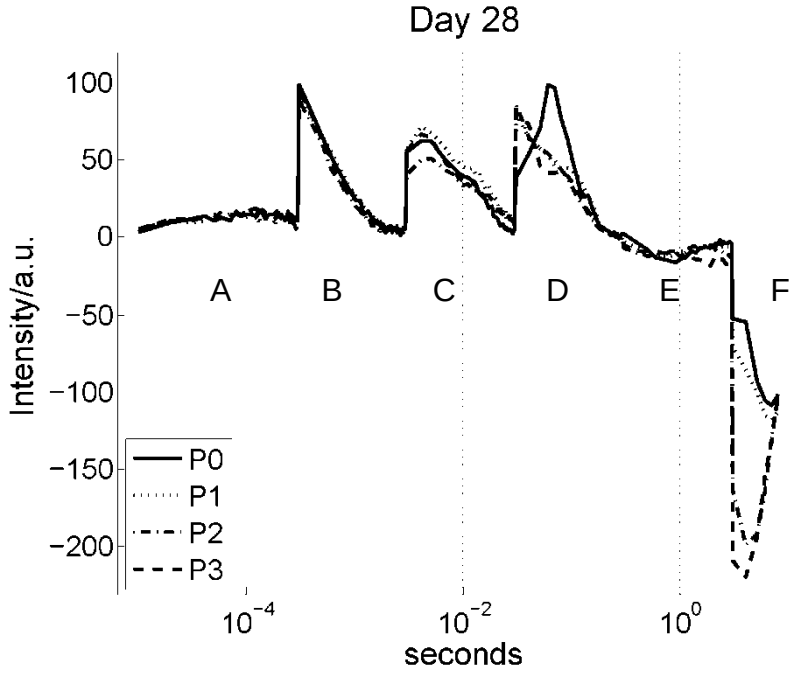

Day 28
100
50
0
−50
−100
−150
−200
Intensity/a.u.
A
B
C
D
E
F
P0
P1
P2
P3
10^{-4}
10^{-2}
10^{0}
seconds

Fig.2

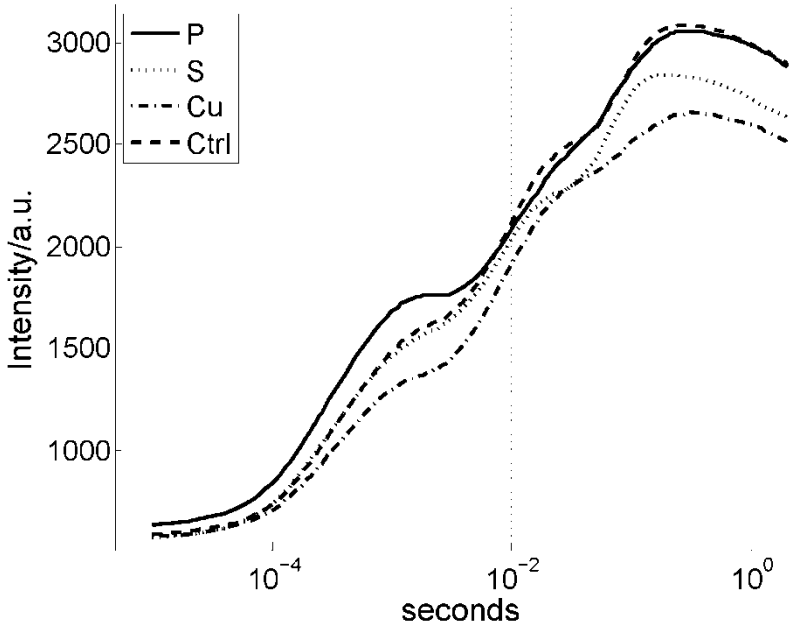

P
S
Cu
Ctrl
3000
2500
2000
1500
1000
Intensity/a.u.
10^{-4}
10^{-2}
10^{0}
seconds

Fig.3

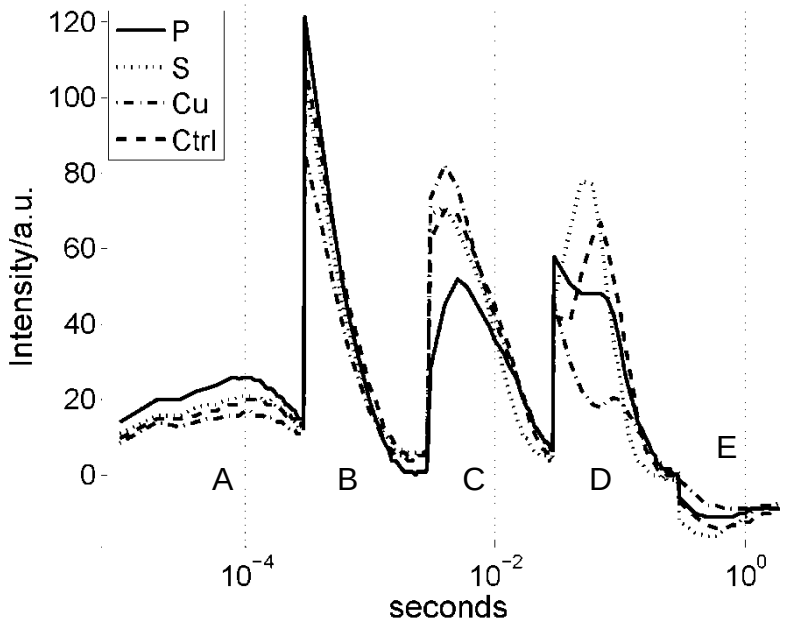

P
S
Cu
Ctrl
120
100
80
60
40
20
0
Intensity/a.u.
A
B
C
D
E
10^{-4}
10^{-2}
10^{0}
seconds

Fig.4

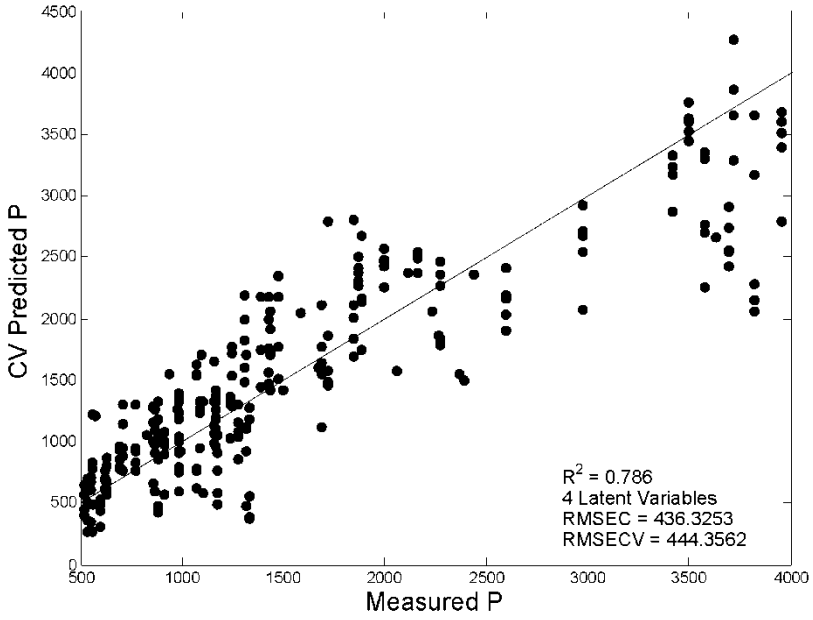

CV Predicted P
Measured P
R^2 = 0.786
4 Latent Variables
RMSEC = 436.3253
RMSECV = 444.3562

Fig.5

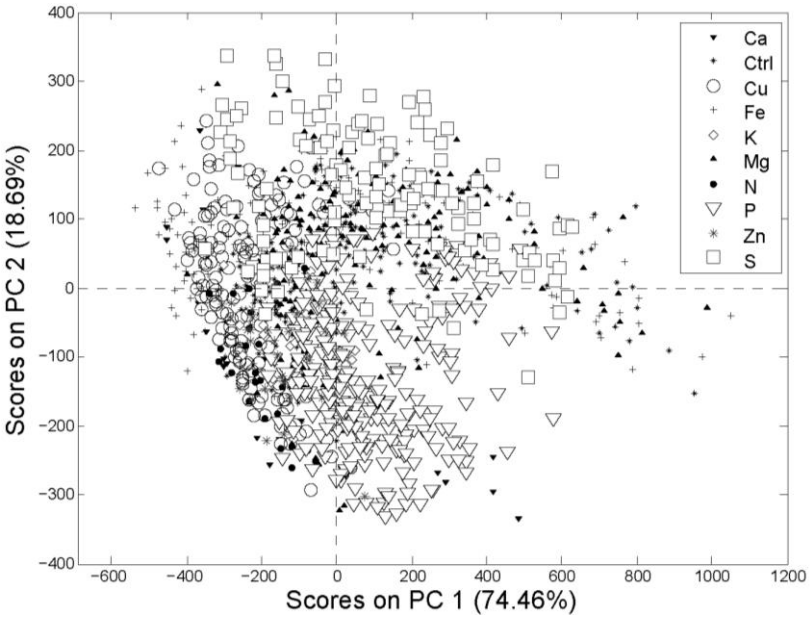

Scores on PC 2 (18.69%)
Scores on PC 1 (74.46%)
Ca
Ctrl
Cu
Fe
K
Mg
N
P
Zn
S

Fig.6