Doctoral Thesis

Establishment and application of phenotyping methods to measure leaf and canopy growth in the laboratory and field

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Establishment and application of phenotyping methods to measure leaf and canopy growth in the laboratory and field

A thesis submitted to attain the degree of
DOCTOR OF SCIENCES of ETH ZURICH
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presented by

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The aim of this thesis was to develop, improve and apply phenotyping methods to measure plant growth on different organizational levels from the single leaf to the plant stand. Growth is a good indicator for the performance of a plant in a given environment. Therefore, the methods applied in this thesis could be beneficial to select genotypes adapted to future environmental conditions.

In a first study (chapter II), a new marker-based tracking approach was developed and tested to analyze two-dimensional leaf expansion at a high temporal resolution. The method is based on beads that are attached to the leaf margin and that serve as artificial landmarks. Leaf expansion is indicated by an increase of the polygon area defined by the beads. So far, fluctuating illumination conditions often caused major problems in approaches in which natural structures of leaves were tracked. This restriction was eliminated by using the beads as artificial landmarks. Leaf growth measurements of soybean were conducted in the field and under controlled conditions in a greenhouse and a climate chamber and successfully analyzed with the new tracking approach.

Leaves of dicotyledonous plants show pronounced diel (24 h) growth patterns. To date, however, it is still uncertain, whether diel leaf growth patterns remain constant throughout the development of a plant. Thus, the newly developed tracking approach was applied in a next study (chapter III), to measure the growth from the primary leaves to leaflets of the seventh trifoliate leaf on soybean plants in a climate chamber. In this study, all measured leaves showed a consistent diel growth pattern with maximum growth towards the end of the night. As a result of the consistent growth pattern, it would be possible to monitor the stress level of a plant via the growth rate on any leaflet.

In the next study (chapter IV), a few properties of the setup described in the first study (chapter II) were adapted to enable the measurement of leaf growth in the field even under rainy conditions. With this adapted setup, leaf growth of soybean was measured in the field and data were analyzed with the tracking approach developed in the first study (chapter II). Measurements in the field revealed highest growth in the afternoon and coinciding patterns of growth rate and air temperature. This is in contrast to the opinion in literature on the diel
leaf growth patterns of dicot plants. Thus, the temperature regime in a climate chamber was modified in a way that mimicked field conditions as good as possible to study the effect on the diel leaf growth pattern of soybean. Under these conditions, a diel leaf growth pattern very similar to that observed in the field occurred. This pattern reverted to a pattern previously described in literature with maximum growth in the early morning, when temperature conditions were reverted to commonly used climate chamber settings. The difference between the two utilized temperature regimes were small but the diel growth pattern changed drastically. Thus, a modified temperature regime most probably also affects underlying control processes of metabolism and gene expression in a plant. In future studies, it is therefore very important to use most realistic climate conditions if such studies are conducted under controlled conditions and aim to predict the performance of plants in the field. Temperature regimes and especially night temperatures need to be adjusted more precisely to better understand their effect on plant growth, metabolism and gene expression. This study may have revealed a key factor how to improve bridging the gap between laboratory and field research in plant science.

For assessing the performance of plants not only growth of single leaves and single plants are important. The growth of the plant community as a plant stand in the field in the end is crucial for the achieved yield and thus the resulting agricultural production. Therefore, in the last study (chapter V) a method using terrestrial laser scanning (TLS) to measure canopy growth in the field was developed, applied and evaluated. The overall aim of this study was to work out the capabilities and the limits of TLS in the field on plot areas of several dozen to hundreds of m² for wheat, maize and soybean. Different filtering approaches were tested to calculate the canopy height from TLS data, from which one proved to be suitable. The TLS method developed in this study enables in a simple way the data acquisition and data analysis of crops in the field. This method can be used for making statements about the development of the canopy height and partially also about plant architectural traits of different crops in the field.

Overall, promising methods to measure plant growth at different organizational levels were developed and tested in this thesis. These methods could be beneficial in the future for breeders to select superior genotypes but also for researchers to investigate different aspects of plant physiology.
Zusammenfassung

Ziel der vorliegenden Doktorarbeit war es, Phänotypisierungsmethoden zu entwickeln, verbessern und anzuwenden, um damit das Pflanzenwachstum auf verschiedenen Ebenen – vom einzelnen Blatt bis zum Pflanzenbestand – zu messen. Wachstum ist ein guter Indikator für die Leistungsfähigkeit einer Pflanze in einer gegebenen Umwelt. Daher könnten die in dieser Doktorarbeit angewendeten Methoden nützlich sein, um Genotypen zu selektieren, welche an zukünftige Umweltbedingungen angepasst sind.


In der nächsten Studie (Kapitel IV) wurden einige Eigenschaften des Versuchsaufbaus, welcher in der ersten Studie (Kapitel II) beschrieben wurde, angepasst, um Blattwachstumsmessungen

Um die Leistungsfähigkeit von Pflanzen abzuschätzen ist nicht nur das Wachstum von einzelnen Blättern und Pflanzen wichtig. Das Wachstum der Pflanzengemeinschaft als Pflanzenbestand im Feld ist schlussendlich entscheidend für den erzielten Ertrag und die daraus resultierende landwirtschaftliche Produktion. Daher wurde in der letzten Studie (Kapitel V) eine Methode unter Verwendung von terrestrischem Laserscanning (TLS) entwickelt, angewendet und evaluiert, um das Bestandeswachstum im Feld zu messen. Das übergreifende Ziel dieser Studie war das Aufzeigen der Möglichkeiten und Grenzen von TLS
im Feld auf Parzellenflächen von mehreren Dutzend bis hundert m² bei Weizen, Mais und Soja. Zum Berechnen der Bestandeshöhe aus TLS-Daten wurden verschiedene Filteransätze getestet, wobei einer davon sich als sehr geeignet herausstellte. Die in dieser Studie entwickelte TLS Methode ermöglicht auf eine einfache Art und Weise die Datenerhebung und Datenanalyse von Kulturpflanzen im Feld. Diese Methode kann verwendet werden, um Aussagen über die Entwicklung der Bestandeshöhe und teilweise auch über die Eigenschaften der Pflanzenarchitektur verschiedener Kulturpflanzen im Feld zu treffen.

# Table of contents

Summary ......................................................................................................................... i

Zusammenfassung ........................................................................................................... iii

I  General introduction .................................................................................................. 1

  1.1 Introduction ........................................................................................................... 2
  1.2 Plant phenotyping ................................................................................................. 2
  1.3 Automated plant phenotyping systems ................................................................. 4
  1.4 Important traits for plant phenotyping ................................................................. 5
  1.5 Plant growth ......................................................................................................... 6
  1.6 Leaf growth measurements .................................................................................. 7
  1.7 Canopy growth measurements ............................................................................ 8
  1.8 Outlook ................................................................................................................. 9
  1.9 Aim and structure of the thesis ........................................................................... 10
  1.10 References ......................................................................................................... 13

II  Diel leaf growth of soybean: a novel method to analyze two-dimensional leaf
    expansion in high temporal resolution based on a marker tracking approach
    (Martrack Leaf) ........................................................................................................ 19

  2.1 Background ......................................................................................................... 21
  2.2 Material and methods ......................................................................................... 24
  2.3 Results ................................................................................................................ 32
  2.4 Discussion .......................................................................................................... 37
  2.5 Conclusion .......................................................................................................... 41
  2.6 References ......................................................................................................... 43

III Diel growth patterns of young soybean (Glycine max) leaflets are synchronous
    throughout different positions on a plant ............................................................... 49

  3.1 Introduction ......................................................................................................... 51
  3.2 Materials and methods ....................................................................................... 52
  3.3 Results ................................................................................................................. 56
  3.4 Discussion .......................................................................................................... 63
  3.5 Conclusion .......................................................................................................... 66
  3.6 References ......................................................................................................... 67
# Table of contents

IV Diel leaf growth pattern of soybean (*Glycine max*) under simulated field conditions: night temperature matters! ................................................................. 69

4.1 Introduction .............................................................................................. 71
4.2 Results ........................................................................................................ 73
4.3 Discussion .................................................................................................. 79
4.4 Conclusions ............................................................................................... 83
4.5 Materials and methods ........................................................................... 85
4.6 References ................................................................................................. 93
4.7 Supplementary material ........................................................................ 96

V Terrestrial 3D laser scanning to track the increase in canopy height of both monocot and dicot crop species under field conditions ........................................ 101

5.1 Background ............................................................................................. 103
5.2 Results ...................................................................................................... 108
5.3 Discussion ................................................................................................. 117
5.4 Conclusion ................................................................................................. 120
5.5 Materials and methods ........................................................................... 121
5.6 References ................................................................................................. 128

VI General discussion .................................................................................... 133

6.1 Leaf growth measurements ..................................................................... 134
6.2 Canopy growth measurements ................................................................. 137
6.3 Applications of phenotyping methods ..................................................... 139
6.4 Conclusions ............................................................................................... 140
6.5 References ................................................................................................. 142

List of abbreviations ..................................................................................... 145

Curriculum vitae ........................................................................................... 147

Acknowledgements ....................................................................................... 149
Chapter I

General introduction
1.1 Introduction

Global agricultural production will be faced with major challenges in the future. Climate change will lead to prolonged growing seasons with less uniform precipitation patterns and increased risk for summer droughts (Solomon et al. 2007). As a consequence, the water availability will be decreased drastically with a strong adverse effect on plant growth and consequently on agricultural production. Furthermore, the pressure of pests, pathogens and weeds in agroecosystems will change due to altered environmental conditions and force agricultural research to find solutions to ensure a sufficient global agricultural production (Nelson et al. 2009).

In addition to challenges by global climate change, a tremendous growth increase of the world population will lead to a vastly increased global food demand (UN 2015, Tilman et al. 2011). Furthermore, arable land usable for food production is in a strong competition with biofuel production. Degradation, salinization and erosion of arable land are problems that lead to reduced soil fertility. Therefore, global food production is facing a big problem: more food has to be produced under less optimal environmental conditions and on less land as in present times. Hence, in the future, improved yields are needed. But not only higher yields in terms of quantities are needed; also more stable yields under different environmental conditions are needed, in other words yield security will be of major importance. Plants have to produce adequate yields under unfavorable environmental conditions that include both abiotic and biotic factors. To achieve this, plant phenotyping with its methods is needed to select genotypes that meet these requirements and thereby to help satisfy the demanded food production. It will be of major importance to conduct precise experiments with respect to exact 24-h fluctuation of temperature.

1.2 Plant phenotyping

Plant phenotypes result from the interaction of genotypes with a multitude of environmental factors (Dhondt et al. 2013). In agricultural production, crop management practices can also affect plant phenotypes. The resulting plant phenotypes can be
characterized by plant phenotyping that ‘refers to a quantitative description of the plant’s anatomical, ontogenetical, physiological and biochemical properties’ (Walter et al. 2015).

In traditional breeding approaches that are still widely applied, plant phenotyping is performed with the breeder’s eye (Studer et al. 2007, van Bueren et al. 2003). In such approaches, for each genotype (crossing) plant vigor and other - in the breeder’s eye - important plant traits are rated by the breeder with the respective extent of subjectivity. Nevertheless, this traditional breeding approach and other improvements by the “Green Revolution” (Borlaug 2000) have achieved enormous increases in yield during the last century. By the introgression of “reduced height genes” in wheat, dwarf and semi-dwarf varieties have been made to stay shorter i.e. produce less straw but on the other hand have higher yields and increased harvest indices. The increased yield was also due to the crossbreeding with disease-resistant varieties. The availability of relatively cheap mineral fertilizers and new synthetic pesticides furthermore strongly contributed to the massive yield increase of cereal grains after World War II. Nowadays, research is paying strong interest on elucidating how breeders perhaps unintentionally altered the root architecture of wheat by the crossbreeding of “reduced height genes” since the “Green Revolution”.

In current agricultural research, plant phenotyping is considered to be the major bottleneck (Furbank & Tester 2011) compared to genotyping, where an immense progress was achieved during the last decades. Genotyping methods are faster and widespread and thus also relatively cheap compared to current plant phenotyping methods. Hence, more weight needs to be given to plant phenotyping and particularly to automated systems to speed up the improvement of plant genotypes for the future. Automated or semi-automated plant phenotyping systems are currently mainly operated under controlled environmental conditions such as in climate chambers or greenhouses. Experiments under laboratory conditions have many well-known limitations. One of the biggest challenges and therefore also the major difference to the field are the environmental conditions. With today’s technologies, it is still very difficult to mimic under controlled conditions the abiotic and the biotic factors that naturally exist in the field. Plant cultivation under controlled conditions is typically being performed in pots and thus has many growth restrictions (Passioura 2006a). Further, the monitored growth periods are
often too short for a precise prediction about a resulting yield. The above mentioned restrictions and many more point out that laboratory results cannot easily be linked to field situations, if at all. Therefore, it is very important to intensively link laboratory and field experiments to allow a better assessment of the significance of laboratory-based results for the field.

1.3 Automated plant phenotyping systems

In recent times, many different automated and semi-automated systems were established at different laboratories worldwide such as “The Plant Accelerator” at the Australian Plant Phenomics Facility or “PHENOVISION” at Ghent University. Many systems are prototypes or based on commercially available systems (e.g. “Scanalyzer” by Lemnatec in different configurations or “FieldScan” by Phenospex). In most of the systems, the same types of sensors are used to quantify plant architecture, plant height, plant growth, plant vigor or other plant characteristics. Different types of visible and near-infrared cameras, multispectral point sensors and different devices to quantify photosynthesis by gas exchange or chlorophyll fluorescence are used for this. By using thermal cameras the water demand related to transpiration and canopy temperature is quantified. Most of the so far established systems are under controlled conditions or semi-field conditions and thus phenotypic data recorded from genotypes in such systems still cannot precisely predict the performance of these genotypes under field conditions. However, established phenotyping technologies are more and more operated in the field from different carriers such as tractors (Montes et al. 2011), airships (Liebisch et al. 2015) or unmanned aerial vehicles (UAVs) (Zhang et al. 2013) in a next generation of phenotyping platforms (Walter et al. 2015). Further, more phenotyping platforms use a combination of multiple, remote sensing-based imaging and non-imaging technologies to quantitatively describe the performance of plants during their development (Walter et al. 2015).

Our research group for crop science is currently establishing the “Field Phenotyping Platform” (FIP) at the field site of the research station for plant science of ETH Zurich in Eschikon, Lindau (Switzerland). Different sensors are mounted on a rope suspended carrier system (spidercam®) which can be automatically positioned on any position of an
area of around one hectare where different crops are planted on small plots under field conditions. Visible, near-infrared and thermal cameras, multispectral point sensors and a laser scanner are currently operated on the FIP.

1.4 Important traits for plant phenotyping

In plant science and traditional breeding approaches a bunch of different plant traits are measured and rated for a long time. Hence, just a few important examples will be given in the following. Plant height for example is strongly related to biomass and thus can be used to estimate biomass (Tilly et al. 2014). Different leaf area traits such as the leaf area index (LAI) or the leaf area density (LAD) and their development are used to describe plant performance, make management decisions or to calculate the light penetration into the canopy (Gladstone & Dokoozlian 2003, Gebbers et al. 2011). Canopy cover can be used to non-destructive assess early vigor-related traits (Mullan & Reynolds 2010, Grieder et al. 2015). The above mentioned and further traits will also be of major importance in automated plant phenotyping systems.

A continuous measurement of plant traits throughout crop development is needed (Walter et al. 2015). Automated plant phenotyping systems can easily do repeated measurements in short time intervals. Thus, the temporal and the spatial resolution that so far often was quite restricted can be improved strongly by such automated approaches. This is just one big advantage of automated systems compared to manual measurements. Furthermore, automated systems should objectively measure plant traits. This, in contrast to manual measurements and ratings where the risk of bias in the data always exists. For rating this risk might be higher than for measurements.

Plant phenotyping includes the measurement and characterization of many different structural, physiological and performance-related traits (Dhondt et al. 2013). Plant phenotyping not only includes the proximal sensing and imaging during the season but also the laboratory analysis of plant samples during the season or near-infrared spectroscopy (NIRS) deployed on harvestable parts of crops at the laboratory but also on machinery during harvest (White et al. 2012, Araus & Cairns 2014). NIRS evaluates spectral features related to physical and chemical characteristics of the samples that allow the
determination of for example the protein, nitrogen, starch or oil content of seeds (Montes et al. 2007, Araus & Cairns 2014). Thus, the analysis of plant samples complements the direct phenotyping under field conditions (Araus & Cairns 2014). However, in recent times, the term phenotyping has more often been linked to non-destructive optical analyses of plant traits based on images and thus is turning its focus back to the initial meaning (Walter et al. 2015).

In our research group, mainly image-based phenotyping technologies are used to measure and characterize growth and architecture of plants during their ontogeny. Hence, in the following I will focus on plant traits derived from imaging technologies. Growth can be measured on different organizational levels on a plant ranging from the single cell to tissue, organ (e.g. a leaf), whole plant to finally the canopy consisting of a community of plants (Dhondt et al. 2013). In this thesis, I investigated the growth of single leaves, growth of leaves on different positions on the same plant and growth of the plant stand.

1.5 Plant growth

A plant is growing by cell division and cell expansion. Plant growth is defined as the irreversible increase in biomass and it is dependent on nutrients, water, CO₂, temperature and on energy from the sun light that is absorbed by different molecules in the chloroplast during photosynthesis. Different environmental parameters can have varyingly strong effects on plant growth and even on the growth pattern. Temperature (Körner 2008, Parent & Tardieu 2012) and water availability (Chaves et al. 2003, Passioura 2006b) are abiotic factors that strongly affect plant growth. Air temperature and root temperature (Poiré 2010a) can have strong effects on the diel growth pattern. Soil water potential and transpiration largely induce cell turgor that in turn is the main driver of leaf expansion (Pantin et al. 2011). The temporal dynamic of growth is strongly influenced by short-term changes of water availability, cell turgor or transpiration (Walter et al. 2009).

Furthermore, the organization and placement of growth zones in leaves can affect leaf growth (Ruts et al. 2012a). In growing leaves of monocot species the zones of cell expansion and photosynthesis are separated, whereas in dicot species the growing leaf tissue is already engaged in photosynthesis (Ruts et al. 2012a). Moreover, growth processes, gene expression and plant metabolism are tightly controlled by internal

Growth rates on different organizational levels on a plant are suitable for the assessment of the capability of a plant to perform well despite environmental factors are – from an agricultural perspective – present in non-optimal conditions during a certain period. Genotypes in breeding programs may differ in their growth reactions and resulting yields to such non-optimal conditions such as drought or chilling temperatures. Therefore, it is important to develop sensitive growth measurement methods and provide these methods to breeders, so that they can select genotypes that can cope best with such non-optimal conditions. Moreover, sensitive growth measurement methods could be used for integrative molecular analyses of growth regulatory networks (Wuyts et al. 2015).

1.6 Leaf growth measurements

Leaf growth reacts very sensitive on changing environmental conditions. Therefore, short-term leaf growth analyses allow for the assessment of the tolerance of a plant to abiotic stress such as drought (Banziger et al. 1999), heat (Lipiec et al. 2013) or nutrient deficiency (Walter et al. 2009). Leaf growth of a wide range of plant species, related and not related to human nutrition has been investigated since the last century (e.g. Avery 1933, Boyer 1970, Walter & Schurr 1999, Wiese et al. 2007). Many of these investigations were carried out to gain information about important agronomical plant traits and partially also to study plant physiology. The used methods largely differed in their spatial and temporal resolution. First measurements in dicot species were performed manually with the help of a ruler or similar devices and thus temporal resolution was limited (Bunce 1977; Randall & Sinclair 1989). First measurements of monocot species i.e. the measurement of the leaf elongation rate (LER) were taken with classical auxanometers (Bovie 1912, Ranson & Parija 1955). Later technical improvements led to the usage of so-called linear voltage differential transducers (LVDTs) (Gallagher 1976, Körner & Woodward 1987, Ben-Haj-Salah & Tardieu 1995) and rotary resistance transducers (RRTs) (Poiré et al. 2010b). In newer approaches, leaf growth of dicot species was measured at a high temporal resolution with the help of image-based methods (e.g. Schmundt et al. 1998, Ainsworth
et al. 2005, Poiré et al. 2010a). In this so-called digital image sequence processing (DISP) approach, leaf growth was measured two-dimensional by analyzing an image sequence of a growing leaf (Schmundt et al. 1998). However, a big disadvantage of this method is that it is highly sensitive to fluctuating illumination conditions. Therefore, this technique is almost not applicable outside of climate chambers such as in a greenhouse and in the field. Hence, there is still a lack of methods to analyze leaf area growth at a high temporal resolution under field conditions.

1.7 Canopy growth measurements

For assessing the performance of plants not only growth of single leaves and single plants are important. Canopy growth gives information about the growth of a plant community in the field, what finally is the place where agricultural production takes place. Besides before mentioned factors, canopy growth is affected by crop management factors such as plant density or plant spacing but also by canopy microclimate and others. Different technologies exist to measure the height of crops in the field. In a very simple approach, images were taken with a commercial digital camera in a rice (Oryza sativa) field to monitor plant height changes (Sritarapipat et al. 2014). In another approach, plant height and other morphological parameters of maize (Zea mays) were measured by a so-called “light-curtain system” (Montes et al. 2011). In these approaches spatial resolution was often restricted or measurements could only be conducted to a certain canopy height. In more complex approaches three-dimensional (3D) images of plants are reconstructed. This for example can be done by using stereo cameras (Biskup et al. 2007) or by analyzing images taken from different viewing angles (Paproki et al. 2012). A more sophisticated technology is the active remote sensing laser rangefinder also known as terrestrial laser scanning (TLS). This technology uses a laser beam to determine the distance to objects. Measurements by TLS lead to a so-called point cloud that depicts a 3D view of the scanned surrounding. From this point cloud of for example a crop field, canopy height, architectural traits or other plant parameters can be obtained. So far, TLS was mostly applied in studies about forest ecology (Parker et al. 2004, Hosoi & Omasa 2009). Measurements of orchard volume (Rosell et al. 2009) or leaf area in viticulture (Arno et al. 2013) have been conducted in the context of precision agriculture. By discriminating
between maize plants, weeds and soil, TLS was applied in an interesting approach for a targeted application of herbicides (Andujar et al. 2013). In phenotyping approaches, TLS was for example applied to investigate morphological parameters such as canopy height (Lumme et al. 2008, Tilly et al. 2014) or leaf area (Gebbers et al. 2011, Hosoi et al. 2011). So far, TLS for field research is still limited since it was often conducted on single plants in pots (Paulus et al. 2014) or on plants like Arabidopsis thaliana (Kaminuma et al. 2004) that do not contribute to agricultural production for human needs. These TLS measurements were often carried out under controlled conditions or if they were conducted in the field it was done on small areas or with low spatial and/or temporal resolution. Thus, there is still the need to improve TLS approaches to measure canopy growth of crops at a high spatial and temporal resolution under field conditions.

1.8 Outlook

Establishment and application of new high-throughput plant phenotyping methods that can be used – ideally on automated systems – in the field under natural conditions and not only under laboratory conditions is very important. Experiments and measurements need to be done in the field because often the transfer of results from the laboratory to the field is difficult or even not possible. If results achieved by plant phenotyping are intended to be used for breeding, they should ideally also be correlated with genetic information such as quantitative trait loci (QTL) for selection by marker-assisted selection (MAS) techniques (Tester & Langridge 2010, Walter et al. 2012). Further, it is crucial that environmental conditions are always included in the assessment of phenotypic data to analyze genotype x environment interactions. By this means, the performance of plant genotypes for the future can hopefully be improved in a faster way. In addition to the selection of genotypes with increased resource use efficiency (Fiorani & Schurr 2013) in breeding programs, a sustainable intensification of agricultural production is needed to meet the increased global food demand. This can among others be achieved by the application of appropriate plant phenotyping methods to improve crop management practices.

Without the constant development of methods to measure and characterize plants in the past, our understanding of plant physiology would not be as it is today and current crop
varieties would not be as productive as they are. Therefore, a continuous investment in developing new methods is essential, although at a first view the necessity of such methods often seems to be questionable.

1.9 Aim and structure of the thesis

Growth rates on different organizational levels are suitable for the assessment of the capability of a plant to perform well despite environmental factors are – from an agricultural perspective – present in non-optimal conditions during a certain period. Thus, methods to measure growth at different organizational levels are important for the selection of genotypes adapted to future environmental conditions or specific local climates. Therefore, it was the aim of this thesis to establish, improve, apply and evaluate methods to measure plant growth from the single leaf to single plants and finally to the plant stand. In the following, a short description of the conducted experiments and the specific hypotheses will be given for chapters II to V.

1.9.1 Chapter II: Diel leaf growth of soybean: a novel method to analyze two-dimensional leaf expansion in high temporal resolution based on a marker tracking approach (Martrack Leaf)

In this study, a new marker-based tracking approach was established and tested to analyze two-dimensional leaf expansion at a high temporal resolution. An important feature of this approach are beads that are attached to the leaf margin and that serve as artificial landmarks. Leaf expansion is approximated by the increase of the polygon area defined by the beads. So far, fluctuating illumination conditions often caused major problems in approaches where the natural structures of leaves were tracked. This problem is prevented by using the beads as artificial landmarks. Leaf growth measurements of soybean (*Glycine max*) were conducted in the field and under controlled conditions in a greenhouse and a climate chamber. Measurements were analyzed with the new tracking approach.
1.9.2 Chapter III: Diel growth patterns of young soybean (*Glycine max*) leaflets are synchronous throughout different positions on a plant

After the successful testing of the new marker-based tracking approach (described in detail in chapter II), in a next study, the growth from the primary leaves to leaflets of the seventh trifoliate leaf on the same soybean plants was measured in a climate chamber. In most studies in which leaf growth was measured, the position of the measured leaves was chosen at random or the position on the plant was not even mentioned. Therefore, it is still unclear whether diel leaf growth patterns remain constant throughout the development of a plant. Hence, in this study, the hypothesis was tested whether young leaflets on the same soybean plants at different phenological stages display an equal diel growth pattern.

1.9.3 Chapter IV: Diel leaf growth pattern of soybean (*Glycine max*) under simulated field conditions: night temperature matters!

To enable the measurement of leaf growth of soybean in the field even under rainy conditions, a few properties of the setup described in chapter II had to be adapted and most importantly made weatherproof. After these adjustments, leaf growth measurements of soybean were conducted in the field and data analyzed with the tracking approach presented in chapter II. In a next step, the climate conditions present during field measurements were set in a climate chamber to simulate the field conditions and to study the effect on the diel growth pattern of soybean leaflets. Results of the leaf growth pattern from these simulated field conditions were compared to results obtained under “conventional” climate chamber conditions. Additionally, the obtained diel leaf growth pattern under the simulated field conditions was compared with results of the field measurements. This comparison was conducted in the context of the transferability of laboratory-based results to the field.

1.9.4 Chapter V: Terrestrial 3D laser scanning to track the increase in canopy height of both monocot and dicot crop species under field conditions

For assessing the performance of plants not only growth of single leaves and single plants are important. The growth of the plant community as a plant stand in a field in the end is
crucial for the resulting yield. Therefore, a method using terrestrial laser scanning (TLS) was established, applied and evaluated in the field during several seasons. Measurements were conducted in maize (*Zea mays*), soybean (*Glycine max*) and wheat (*Triticum aestivum*). During the growing season 2015, measurements of the soybean and maize field under the Field Phenotyping Platform (FIP) were conducted by TLS from the rope suspended carrier system (data not shown in this thesis). In previous years, the laser scanner was mounted on an elevator tripod that was moved manually to the different scanning positions. In this study, the hypotheses were tested whether TLS is capable to elucidate (1) differences in architecture that exist between genotypes; (2) genotypic differences between canopy growth during the season and (3) short-term growth fluctuations (within 24 h), which could e.g. indicate responses to rapidly fluctuating environmental conditions.
1.10 References


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Chapter II

Diel leaf growth of soybean: a novel method to analyze two-dimensional leaf expansion in high temporal resolution based on a marker tracking approach (Martrack Leaf)

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Additional files can be found in the published version.
Abstract

**Background:** We present a novel method for quantitative analysis of dicot leaf expansion at high temporal resolution. Image sequences of growing leaves were assessed using a marker tracking algorithm. An important feature of the method is the attachment of dark beads that serve as artificial landmarks to the leaf margin. The beads are mechanically constricted to the focal plane of a camera. Leaf expansion is approximated by the increase in area of the polygon defined by the centers of mass of the beads surrounding the leaf. Fluctuating illumination conditions often pose serious problems for tracking natural structures of a leaf; this problem is circumvented here by the use of the beads.

**Results:** The new method has been used to assess leaf growth in environmental situations with different illumination conditions that are typical in agricultural and biological experiments: Constant illumination via fluorescent light tubes in a climate chamber, a mix of natural and artificial illumination in a greenhouse and natural illumination of the situation on typical summer days in the field. Typical features of diel (24h) soybean leaf growth patterns were revealed in all three conditions, thereby demonstrating the general applicability of the method. Algorithms are provided to the entire community interested in using such approaches.

**Conclusions:** The implementation Martrack Leaf presented here is a robust method to investigate diel leaf growth rhythms both under natural and artificial illumination conditions. It will be beneficial for the further elucidation of genotype x environment x management interactions affecting leaf growth processes.

**Keywords:** Marker tracking, Phenotyping, Image analysis, Plant growth, Diel growth, Natural illumination.
2.1 Background

Plant size and shape is determined by its growth, while growth itself can be influenced by numerous endogenous, genetic, epigenetic and environmental factors. It is well known that leaf growth, many metabolic reactions, physiological processes and elements of regulatory networks show diel (24 h) fluctuations that are partly controlled by the circadian clock (Hennessey & Field 1991, McClung 2001, Dodd et al. 2005, Lu et al. 2005, Nozue et al. 2007, Espinoza et al. 2010, Graf et al. 2010, Poiré et al. 2010b, Graf & Smith 2011, Farré 2012, Kinmonth-Schultz et al. 2013, Dodd et al. 2014). Light and temperature beside many other environmental parameters, which can affect that circadian rhythm are the most “potent” and important input factors of circadian entrainment (McClung 2001, Millar 2004, McClung & Gutierrez 2010).

Overall, circadian regulation has to be assumed necessary to maintain plant productivity (Dodd et al. 2014). Growth thus can be considered as the major, integrating output process of plant metabolism, cumulating over time into final leaf size (Horiguchi et al. 2006a, Horiguchi et al. 2006b). Dynamic fluctuations of growth therefore reflect adjustments of endogenous processes to variations of environmental conditions; their elucidation can be of importance to understand processes of biomass and yield formation (Walter & Schurr 2005).

Therefore, it is of vital importance to monitor diel growth patterns as influenced by different environmental conditions (Takami et al. 1982, Taylor & Davies 1985, Roden et al. 1990, Munns et al. 2000, Reymond et al. 2003, Walter et al. 2008, Poiré et al. 2010a, Poiré et al. 2010b, Kjaer et al. 2012, Ruts et al. 2012b), development (Wilhelm & Nelson 1978) or by alterations in metabolism (Wiese et al. 2007, Timm et al. 2012). Several quantitative methods based on digital image processing and sequence analysis have been developed and applied to study fluctuations in growth of various plant organs such as roots and leaves in recent years (Schmundt et al. 1998, van der Weele et al. 2003, Millar 2004, Horiguchi et al. 2006a, Walter et al. 2009, McClung et al. 2010). Other non-imaging methods for measurements of growth, such as “classical auxanometers” (Ruge et al. 1961), linear voltage differential transducers (LVDTs) (Gallagher et al. 1976, Körner & Woodward 1987), rotary resistance transducers (RRTs) (Poiré et al. 2010b), direct
assessment of plant size or subsequent manual assessment of displacement of markings that have been applied to the organ surface (Avery 1933, Walter & Schurr 1999), exist and have been used both in field and climate chamber experiments. Yet they are limited either to measurements of one-dimensional growth (elongation), are labor intensive needing manual processing steps or do not provide a suitable high temporal or spatial resolution, as do quantitative methods based on image processing, which are thus preferable.

In general the techniques applied can be classified in three groups of image processing approaches (Walter et al. 2009): (I) “morphometric”, (II) “optical flow” and (III) “particle / marker tracking” with the two latter methods providing the potential for analysis of spatially differentiating growth or strain rates within the organ if marker or grey value structures within the organ can be followed kinematically.

Morphometric approaches are based on segmentation algorithms calculating projected leaf area and additional shape parameters from analysis of the outline of leaves or leaf rosettes. High-throughput phenotyping methods are typically based on such approaches (Leister et al. 1999, Granier et al. 2006, Walter et al. 2007, Jansen et al. 2009, Arvidsson et al. 2011, De Vylder et al. 2012). Even though morphometric image processing has been applied to estimate the projected area and shape of single leaves both in vivo and in studies of harvested leaves (Taylor et al. 2003) it has been found most effective in investigations on a whole plant/shoot level. For investigation of diel growth patterns, morphometric approaches have found only limited usage due to problems arising from leaf motion (Walter et al. 2009). Furthermore it is not possible to extract spatial differences in growth rate within the segmented plant organs.

Optical flow based growth estimation is the most powerful method to provide both high temporal and high spatial resolution. Such methods have been applied to study leaf (Walter et al. 2005, Matsubara et al. 2006, Matsubara & Walter 2007, Poiré et al. 2010b), root (Walter et al. 2002, Walter et al. 2003, Nagel et al. 2009) and hypocotyl growth (Bergougnoux et al. 2012). The movement of structural patterns within the space-time-cube of subsequent images is used to calculate velocity fields of structural elements such as vein intersections, trichomes or ink dots applied on the leaf surface on a subpixel level of accuracy (Schurr et al. 2001), as long as image brightness is constant. Recent
improvements allowed to study diel growth patterns of small leaves of the model species *Arabidopsis thaliana*, thereby opening the possibility to even investigate alterations in growth of a wide repertoire of mutant and transgenic plants such as starch deficiency, circadian or photorespiratory mutants (Wiese et al. 2007, Ruts et al. 2012a, Timm et al. 2012). Yet, optical flow based approaches are sensitive to brightness fluctuations and they require that structural patterns are not moving too fast from one image to the next. Therefore, even though leaves are growing slowly, a huge number of images have to be acquired within short time, as physical structures are not allowed to change position for more than one pixel between consecutive images. This inevitably increases the size of image sequences. Although computer storage capacity and processing speed continuously advance, this problem should not be underestimated. An image sequence showing expansion of a single leaf throughout several days typically needs to comprise several gigabytes (one image per minute) to allow for optical flow based data evaluation. After all calculation, the amount of data typically exceeds 10 gigabytes. With a project typically consisting of dozens of sequences that have been acquired it is obvious that this leads to challenges in data handling, storage, backup and exchange of data even under today’s norms. The problem becomes even potentiated, if it is necessary to increase the spatial resolution, as characteristically higher spatial resolution makes it necessary to also increase the temporal resolution of image acquisition. A second major problem associated with the optical flow approaches is the fact that the so-called ‘brightness change constraint equation’ (BCCE) has to be fulfilled throughout the sequence, which means, that a constant brightness has to be assured (Horn & Schunck 1981, Haussecker & Fleet 2000, Spies et al. 2000, Schuchert & Scharr 2009). Under controlled conditions in climate chambers this is possible throughout day and night by using infrared diode illumination of the scenery and infrared bandpass filters in front of the camera. Yet, under greenhouse conditions and even more in the field, it is nearly impossible to fulfill this intensity requirement due to diurnal fluctuations in illumination.

Marker tracking is a technique of image processing and analysis, in which a discrete number of landmarks is registered initially within the object of interest. The position of these featured landmarks is followed in the consecutive images of an image sequence using pattern matching in the local neighbourhood of these markers. Based on this
approach, it is possible to calculate relative growth rate (RGR) with high temporal resolution (Schurr 1998). Such approaches have been used successfully to study root growth (Ishikawa & Evans 1997, Beemster & Baskin 1998, Basu et al. 2007) by following either artificially applied particles on the root surface or by taking cell walls as markers. In the context of leaf growth though, marker-tracking-based image processing routines only have been applied in a limited number of studies (Wang et al. 2011, Remmler & Rolland-Lagan 2012). Moreover, in these studies there was no attempt to analyze leaf growth at high temporal resolution or to perform field experiments. In general, marker tracking is a very powerful and robust method in image processing, in which the BCCE requirement does not have to be fulfilled (Lewis 1995). Therefore, it should be possible in principle to assemble a marker-tracking based approach that allows assessment of diel leaf growth patterns in field experiments.

It was the aim of this study to establish a marker-based approach that allows monitoring of diel leaf growth fluctuation in various illumination conditions, revealing typical features of diel leaf growth patterns that are known from optical flow based approaches, without further consideration of base-tip gradients or other spatial growth differences within the leaf lamina.

### 2.2 Material and methods

#### 2.2.1 Growth conditions

Soybean plants (*Glycine max* (L.) Merrill, variety “Gallec”) were grown in plastic pots (10 cm × 10 cm × 10 cm) filled with substrate (“Spezialmischung 209”, RICOTER Erdaufbereitung AG, Aarberg, Switzerland) inside a climate chamber (Conviron, Winnipeg, Canada) under controlled conditions with a 13h/11h light/dark photoperiod: light intensity 580 ± 75 μmol PAR m$^{-2}$ s$^{-1}$; average temperature of 24°C (day) and 20°C (night); relative humidity 60% (day and night). The climate chamber was equipped with a 2:1 mixtures of fluorescent lamps of two types (Master TL5 HO 54W/840, Koninklijke Philips Electronics N.V., Eindhoven, the Netherlands and FHO54W/T5/GRO, Havells Sylvania Europe Ltd, London, UK).
Additional soybean plants (variety “Amphor”) were grown in a greenhouse and in the field of the research station for plant science of ETH Zurich in Lindau- Eschikon. Soybean plants in the greenhouse were grown in plastic pots as described above, filled with substrate (“Spezialmischung 209/09-047”, RICOTER Erdaufbereitung AG, Aarberg, Switzerland). The plants were kept under standard greenhouse conditions and were watered on a regular basis. In the field, plants were sown in small plots (6.5 m length, 1.5 m width, 18 cm row width, 60 seeds/m²).

2.2.2 Mechanical leaf fixation and preparation of image acquisition

One growing leaf of every investigated plant was fixed in the focal plane of a top mounted camera placed above the abaxial leaf surface using 5 small weights of 2.5 to 9 g attached with strings and glue (Pattex® KRAFTKLEBER Classic, Henkel AG & Co. KGaA, Düsseldorf, Germany) to the leaf surface (Figures 2.1 and 2.2) (Walter et al. 2003, Wiese et al. 2007, Poiré et al. 2010b, Timm et al. 2012). Small leaves were fixed using small weights; preliminary experiments showed that the weights did not affect final leaf size or shape. Weights were hung over a circular metal frame around the leaf. An additional weight was used as counterforce attached at the opposite side of the shoot to avoid unwanted movements of the plants. Additionally, parafilm was used to fix the leaf at its base to a thin metal bar in the ring without hurting the plant, thereby assuring that stem elongation did not lift parts of the leaf above the focal plane of the camera during acquisition of the image sequence. Black plastic beads (5 mm diameter) were glued to the strings at the leaf border to provide artificial landmarks that allowed registration of marker movements, see sketch in Figure 2.3.

To allow continuous measurement of plant growth during night and day, a metal ring with six infrared LED clusters (940 nm) was used as illumination source (see Wiese et al. 2007, Timm et al. 2012). Usage of infrared LEDs has two major advantages: (1) leaf growth and plant metabolism are not affected by near-infrared light beyond 800 nm and (2) as leaves
diffusely reflect more light in the infrared region of the spectrum, contrast between leaves and background is enhanced while specular reflexes can be avoided.

Figure 2.1 Setup used in the field. (A) Overview of the soybean field and setup; (B) Setup wrapped in plastic bags due to rain (measurement stopped); (C) Close-up view of the setup with infrared camera on top, infrared diodes and a soybean leaf fixed with strings glued to the leaf with attached weights; (D) Close-up view of the fixed soybean leaf with attached black beads; (E) Original image of a soybean leaf in the field taken with an infrared camera.
2.2.3 Image acquisition

Monitoring and analysis of growth in greenhouses and in the field was performed using a standard progressive monochrome CCD camera (XC-55, Sony Corporation, Tokyo, Japan) linked to a personal computer (PC). Images of the growing leaf were acquired every 90 seconds with a resolution of 640 × 480 pixels. The camera was equipped with a lens (H1214-M, 12 mm 1:1.2, Pentax Ricoh Imaging Co., Ltd, Tokyo, Japan) and a narrow bandpass interference infrared filter (940 nm, Edmund Optics Ltd, York, UK) to improve overall image quality and to allow continuous measurement under artificial near-infrared illumination during night and day with fixed camera settings. Automatic gain correction (AGC) was activated for image acquisition under field conditions. Image acquisition in the near infrared region of the spectrum is generally possible in this setup, as standard CCD cameras typically are sensitive up to a wavelength of 1100 nm if no additional hot-mirror or filter is placed inside the camera. Thus, the overall setup is highly comparable to that of the so-called ‘digital image sequence processing (DISP) setup’ (Schmudt et al. 1998, Walter et al. 2009) that has been used frequently to monitor diel leaf growth patterns via an optical flow based algorithm. Image acquisition in the field was performed on several consecutive days without rain to avoid any hardware defects. No additional shelter was applied.
Image acquisition in the climate chamber was conducted with monochrome CMOS cameras (DMK 23GP031, The Imaging Source Europe GmbH, 28215 Bremen, Germany). Each camera was equipped with a lens (C2514-M, 25 mm 1:1.4, Pentax Ricoh Imaging Co., Ltd, Tokyo, Japan) and a narrow bandpass interference infrared filter (940 nm, Edmund Optics Ltd, York, UK). The cameras were linked via Ethernet cables to a switch (SG300-10P, Cisco Systems Inc., San Jose, USA) and this in turn linked to a router (Cable/DSL Web Safe Router RP G14 v4, NETGEAR Inc., San Jose, USA) and to a PC. Images were acquired every 90 seconds with a resolution of maximally $2592 \times 1944$ pixels and the images were stored directly from the software “IC Capture” (The Imaging Source Europe GmbH, 28215 Bremen, Germany) to a PC. By using a switch, several cameras were operated simultaneously with only one PC. Another advantage of these cameras was that they were powered over Ethernet so no additional power supply was needed.
Figure 2.3 Principle sketch of setup. (A) Top view of soybean leaf fixed with strings in a metal frame with marker beads attached at the leaf margin, the artificial background is shown in gray; (B) Whole setup in top side view with camera, infrared LED clusters and fixed soybean leaf.
Algorithm and software for optimized leaf growth analysis

The algorithm for marker tracking was implemented in Matlab 7.12 (The Mathworks, Natick, MA, USA). Template sizes of 23 × 23 pixels up to 85 × 85 pixels depending on image resolution were tracked in the local neighborhood with a search length of 6 pixels and larger. These parameters can be freely adjusted using a graphical user interface. The small black beads attached to the leaf margin were used as artificial landmarks and were selected by clicking on them in the initial image of the image sequence in the graphical user interface (Figure 2.4).

Based on the calculated area it is possible to calculate relative growth rate (RGR) of the enclosed pentagon for every frame of the image sequence by using the following equation (A$_1$ area at image (frame) number $f_1$):

$$\text{RGR (in \% per frame)} = \frac{\ln A_2 - \ln A_1}{f_2 - f_1} \times 100$$

To calculate RGR in percent per hour for every frame, a time correction factor $f_c$ corresponding to the number of images acquired during every hour was applied:

$$\text{RGR (in \% per hour)} = \text{RGR (\% per frame)} f_c$$

As images were acquired every 90 seconds, a time correction factor of 40 was used throughout the experiment.

The graphical user interface (GUI) implemented in Matlab was kept as simple as possible with processing scheme implemented as shown in Figure 2.4. Initially the recorded image sequence is opened and in the first frame of the sequence the center of each bead is selected separately by mouse-clicks. Afterwards, a template-size around the black beads has to be defined. In the following step, a search length has to be assigned for the neighborhood in which the bead has to be found in consecutively following images (frames). The calculation is then started and every black bead is tracked throughout the whole sequence. At the end of this process, the path of the center of every tracked bead throughout the sequence is displayed with a red line in the starting image (Figure 2.5).
The block matching algorithm determines the best position of each template in the current image by normalized cross-correlation (CC) (Lewis 1995).

$$x, y \in S \quad CC(x, y) = \frac{\sum_{m,n} (i(x + m, y + n) - I_{xy}) \ast (t(m, n) - T)}{\sqrt{\sum_{m,n} (i(x + m, y + n) - I_{xy})^2 \ast \sum_{m,n} (t(m, n) - T)^2}}$$

Where S is the search area, x,y are the upper left coordinates of the image area which is compared to the template, i is the image. $I_{xy}$ is the mean gray value of the image area which is matched to the template t, and T is the mean gray value of t. The coordinates within the matched image area, both of i and the template t, are called m and n.

Template size and search area are adjustable to the image material and temporal sampling of the scene. For each template the CC is calculated around the position in the last investigated image in the chosen search area. The best position is localized by the maximal CC value $CC_{\text{max}}$. If $CC_{\text{max}}$ is lower than 0.7 the template is regarded as not found, which ensures a high quality of template positions.

Subpixel accuracy of template positions is achieved by using a quadratic interpolator for the cross-correlation (Tian & Huhns 1986). The maximum of the quadratic polynomial defined by $CC_{\text{max}}$ and its neighboring CC values in x-direction therefore gives the subpixel position in x-direction; the subpixel position in y-direction is determined accordingly.

For each frame, the bead positions define a polygon, which approximates the leaf area. The area of the polygon is calculated with the Gauß-formula for trapezes:

$$A = 0.5 \sum_{i=1}^{n} y_{i+1}x_{i} - y_{i}x_{i+1}$$

Where A is the polygon area, x,y are the coordinates of the n corner points of the polygon. Indices are regarded modulo n, which means $x_{n+1} = x_{1}$.

Martrack Leaf is provided online compiled for different operating systems (for detailed manual see Additional file 1, linux see Additional file 2, Mac see Additional file 3 and Windows 64-bit see Additional file 4). In order to execute Martrack Leaf, the Matlab Compiler Runtime (MCR; Vers. R2012a 64-Bit) (The Mathworks, Natick, MA, USA) needs
to be installed on the user machine (download at www.mathworks.com/products/compiler/mcr/). Files with extension .fig are figures in a Matlab-specific format, they can be displayed, printed and saved using the provided executable showfigure.exe (included in the Additional files for each operating system).

2.3 Results

In preliminary experiments, different wooden and plastic beads from local crafts stores were tested for their optical properties. Most of the black beads tested fulfilled the requirement to show a low reflectance in the near-infrared range. Therefore, it was easy to differentiate beads from the leaves and leaf margins. Nevertheless, soil and nonsoil shadowed backgrounds also often appeared very dark, which made it impossible to maintain contrast differences between beads and background under many conditions.

It was therefore beneficial to introduce a bright background below the leaf, thereby increasing contrast between beads and background (see Figures 2.1 D+E and 2.2). Experiments performed in climate chambers showed clearly oscillating temporal leaf growth patterns (see Figure 2.6) RGR increased during the day, reaching a maximum at day-night-transition. At night, RGR declined again. Short-term fluctuations of RGR were present, but the overall diel pattern remained constant throughout several days.
Figure 2.5 Illustration of the growth of a soybean leaf in a climate chamber. The five red lines are overlaid to the first image of a sequence. They indicate the path of the center of every single black bead tracked over a period of 86.5 hours with Martrack Leaf. The green pentagon shows the leaf area at beginning of the measurement whereas the yellow pentagon denotes the area at the end of the measurement.

In the field, similar diel leaf growth patterns were obtained (Figure 2.7). At noon though, plants grown in the field showed a pronounced, transient drop in their RGR. Fixation of leaves in the field worked well, and movement of leaves was prevented by leaf fixation even when relatively strong gusts of wind were present. In a period of dry weather, it was therefore possible to monitor leaf growth on three consecutive days without rain. Since the setup and outdoor computer installation was not waterproof, care was taken to protect it from eventually upcoming rain if necessary (Figure 2.1B).

In greenhouse experiments, maximal growth was also observed at night and the overall growth patterns were similar to the patterns obtained in the climate chambers and in the field (Figure 2.8). Yet, under these conditions, secondary fluctuations of leaf RGR occurred at night with a phase length of two to three hours.
Figure 2.6 Diel growth pattern of soybean leaves in the climate chamber. (A, B, C) Relative growth rates (RGR) of three soybean leaves from day 1 to day 4. (D) Relative humidity (%) and (E) temperature (°C) throughout the measurements.
Figure 2.7 Diel growth pattern of soybean leaves in the field. (A, B) Relative growth rates (RGR) of two soybean leaves on day 1 and day 2; (C) global radiation (Wh/m²); (D) relative humidity (%) and (E) temperature (°C) throughout two days in the field.
Figure 2.8 Diel growth pattern of soybean leaves in the greenhouse. (A, B, C) Relative growth rates (RGR) in % per hour of three soybean leaves for day 1-3 (A, B) and day 1 and 2 (C); (D) Global radiation (Wh m$^{-2}$); (E) relative humidity (%) and (F) temperature (°C) for corresponding days.
Light intensity in the climate chamber was kept constant at around 580 ± 75 μmol PAR m\(^{-2}\)s\(^{-1}\) during the light period and it was completely dark during night (data not shown). In the field, global radiation increased continuously beginning at around 4 am and reached its peak at noon but then started again to decline towards dusk (Figure 2.7). At both measuring days, a temporary drop in global radiation before noon was observed in the field. In the greenhouse, a similar pattern as seen in the field occurred with a drop in global radiation in the middle of the morning (Figure 2.8).

Relative humidity in the climate chamber was kept constant at 60% with no strong fluctuations during all four days (Figure 2.6). In the field, relative humidity was highest during night and decreased continuously during the day reaching its minimum value in mid-afternoon (Figure 2.7). In greenhouse experiments, relative humidity was kept constant at around 60%, comparable to climate chamber experiments (Figure 2.8).

Temperature in the climate chamber was kept at 24°C during the light period and at 20°C during night (Figure 2.6). Temperature at the field site fluctuated more severely compared to greenhouse and climate chamber conditions, reaching a maximum in mid-afternoon (Figure 2.7). In greenhouse experiments temperature showed no strong fluctuations and was kept at roughly 22°C (Figure 2.8).

2.4 Discussion

Diel growth patterns Image analysis of soybean leaf growth based on the Martrack Leaf algorithm was shown to be robust under indoor and outdoor conditions. The basic pattern of diel leaf growth was comparable to patterns described before, which were analyzed by optical flow based approaches (Ainsworth et al. 2005). Based on the few data series acquired here, it is not possible to conclude on the statistical significance of the differences of the diel growth cycles obtained under the different illumination conditions. The focus of the manuscript is to show that the method produces meaningful results even under very different illumination conditions. Nevertheless, it is important to point out that similarities and differences between treatments with respect to the resulting diel leaf growth cycles are physiologically reasonable. The observed midday leaf growth depression in the field, for example, might be a short-term stress reaction, which could
have been linked to a general water vapor deficit, or to adaptations in transpiration rates under full sun exposure. It is well-known that short-term alterations in turgor pressure lead to short-term growth peaks or troughs (Schmundt et al. 1998, Walter et al. 2005). At this time of the day, also global irradiation decreased transiently, suggesting another possible reason for the deviation of leaf growth in the field from the smoother pattern observed in the climate chamber. This has to be investigated in more detail in future studies. In the greenhouse, growth rates were rather low and highest growth activity was observed during the night. Again, the overall diel growth pattern was comparable to the pattern described previously (Ainsworth et al. 2005) and it did not show a direct relation to air temperature but seems to be rather dominated by endogenous control mechanisms. It is principally possible, that those differences are related to differences in leaf and plant age: Plants investigated in climate chamber, greenhouse and in the field experienced different environmental conditions throughout their entire development. Therefore, it was not possible to compare growth of leaves of identical plant developmental stages. To date, there is no indication in literature, how severely differences in plant development might potentially affect diel leaf growth patterns.

2.4.1 Comparison to other methods

Martrack Leaf is more robust than optical flow based approaches and provides higher experimental versatility compared to morphometric analyses or to mechanical analyses of leaf elongation growth – such as linear variable displacement transducer (LVDT) or rotary resistance transducer (RRT) approaches (Additional files 5 and 6: Tables S1 and S2). Compared to optical flow analysis, marker tracking allows for larger movements of the tracked structures. Markers can be tracked in consecutive images as long as they move less than the selected search length. To circumvent confounding of different markers (beads) the search-length should be chosen smaller than the closest distance between two markers.

In optical flow analysis, movement of structures must not exceed one pixel per frame. Martrack Leaf allows image analysis with fewer images acquired within 24 h to reveal basic diel leaf growth patterns compared to optical flow based procedures such as DISP.
Moreover, wind-induced shifts of the leaf from one image to the next do not pose a severe problem to Martrack Leaf.

Another reason, why optical flow based approaches do not provide high chances for successful leaf growth analyses in the field is the requirement of the BCCE. Only under special circumstances, such as practically cloudless days (Walter et al. 2005), image brightness changes are slow enough to provide constant brightness throughout a large stretch of the image sequence. To avoid brightness changes within image sequences, automatic adjustments to image brightness are typically used in industrial and consumer image acquisition. This can be realized for example by Automatic Gain Correction (AGC) which can be applied to either the complete image or to a selected region of an image or object. Yet for growth analysis based on grey value intensities, such as in the optical flow based DISP method, AGC usage causes additional problems and is thus typically avoided by using manually fixed set values.

In typical image sequences of growing leaves, brightness changes often occur heterogeneously for example in the form of shadows from neighboring leaves or technical structures that travel slowly through the image sequence or that increase in size. These brightness gradients can disturb the quality of an image sequence severely and they can lead to unwanted artifacts in the calculation of RGR. If these artifacts are corrected for automatically, the relation between brightness of neighboring structures is shifted, which leads to the situation that those structures cannot be followed correctly. Biases can also be caused by changes in the reflectivity of the background: Soil for example normally shows a very low reflectivity in the near infrared part of the spectrum but its reflectivity increases with reduced water content.

Optical flow based methods provide advantages with respect to the spatial differentiation of growth rates within the analyzed leaf and they often do not require application of marks to the leaf surface. It also has to be pointed out that brightness changes can lead to problems also in marker tracking approaches since they also hamper marker recognition: Tracking of the marker structures in Martrack Leaf is based on block matching algorithms. Block matching algorithms are frequently used in motion estimation especially for video compression (Barjatya 2004, Huang et al. 2006, Wei et al. 2008) or other
applications such as the evaluation of microscopic images (Cesa et al. 2007). As the block matching algorithm compares image patches, any change of the compared patches due to occurring or moving shadows lowers the accuracy of the position determination. This could be circumvented by a template matching algorithm which will be implemented in further work. Furthermore, the chosen template size and search length as well as the image resolution of the sequence can have an effect on the analysis by the algorithm. Thus, the template size should be chosen in a way that allows for the bead to fit inside the template, but the template size should not be selected markedly larger than the bead. The search length needs to be chosen in a way that the bead can be tracked during the whole sequence. Block matching algorithms could also be implemented in optical-flow based techniques, but this is cumbersome and would increase computing time and would not give any advantage as long, as the growth of whole organs is regarded.

2.4.2 Issues related to storage and image processing times

One typical difference in practical handling of the different methods used for diel leaf growth analysis is related to the amount of storage and processing and evaluation time necessary for calculations and long term backup. Typically RRTs provide the fewest problems in this respect. Acquired data stacks are very small, even if acquisition is performed in very high temporal resolution. In contrast the DISP method produces enormous stacks of data for every plant investigated. Image sequences of growing leaves acquired for DISP analysis over several days characteristically have a size of 1 to 2 GB depending on the selected image resolution, bit depth, time between frames and the overall duration of image acquisition. If higher temporal resolution is necessary (since leaves are otherwise growing too rapidly to fulfill the requirement of a maximal velocity of 1 pixel per frame), or if plants are monitored for many consecutive days, sequences can easily excess sizes of 2 GB. Image sequences acquired for DISP are very big and their evaluation requires creating of several other files that contain the information on velocities of all image pixels in x- and y-direction and on quality estimations. These additional files exceed the size of the original image sequences. Thus, overall size of image sequences for each investigated plant/leaf typically lies in the range of 5 to 8 GB. An experimental design with several replicates under different environmental conditions or
mutant lines easily can be in the range of 100 to 1000 GB if standard camera resolutions (640 × 480 to 800 × 600 pixels) are selected.

2.5 Conclusion

Robust tracking based methods such as Martrack Leaf will be beneficial for the further elucidation of genotype × environment × management interactions affecting leaf growth processes. Thereby, they will be able to play an important role both for the elucidation of processes controlling leaf growth and for an improved understanding of growth reaction to the variation of environmental parameters alike.
Competing interests
The authors have no competing interests to declare.

Authors’ contributions
NK designed the measurement strategy and implemented Martrack Leaf in Matlab. Pre-experimental beta testing of the algorithm implementation was performed by MM, MF and NK. MM drafted the manuscript with help and contributions by MF, NK and AW. MM, MF and AW designed and performed experiments for leaf growth analysis in field, greenhouse and climate chambers. Growth analysis of soybean leaves using Martrack Leaf was performed by MM and MF with figure plates and eventually created and layouted using Sigma Plot 12 and Corel Draw X4. All authors read and approved the final manuscript.

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2.6 References


Chapter III

Diel growth patterns of young soybean (Glycine max) leaflets are synchronous throughout different positions on a plant

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¹Supplemental data can be found in the published version.
Abstract

Leaf growth is controlled by various internal and external factors. Leaves of dicotyledonous plants show pronounced diel (24 h) growth patterns that are controlled by the circadian clock. To date, it is still uncertain, whether diel leaf growth patterns remain constant throughout the development of a plant. In this study, we followed growth from the primary leaves to leaflets of the seventh trifoliate leaf of soybean (Glycine max) on the same plants with a recently developed imaging-based method under controlled conditions and at a high temporal resolution. We found that all leaflets displayed a consistent diel growth pattern with maximum growth towards the end of the night. In some leaves, growth maxima occurred somewhat later – at dawn – as long as the leaves were still in a very early developmental stage. Yet, overall, diel growth patterns of leaves from different positions within the canopy were highly synchronous. Therefore, the diel growth pattern of any leaf at a given point in time is representative for the overall diel growth pattern of the plant leaf canopy and a deviation from the normal diel growth pattern can indicate that the plant is currently facing stress.

Keywords: Image analysis, leaf growth, phenotyping, marker tracking, circadian, drought, development, growth.
3.1 Introduction

Leaf growth is controlled by many internal and external cues (Nozue et al. 2007). The typical diel (24 h) fluctuations in leaf growth are regulated by various signalling and physiological processes that are partly modulated by the circadian clock (McClung 2001, Farré 2012, Ruts et al. 2012). In particular, the photosynthetic apparatus is coupled to the circadian clock by different signalling pathways (Dodd et al. 2014). As an example, by introducing the Arabidopsis thaliana B-box domain gene AtBBX32, changes in yield of soybean (Glycine max) correlated with changes in clock gene expression (Preuss et al. 2012). Non-invasive, optical leaf growth analysis offers a unique opportunity to quantify short-term growth fluctuations and to elucidate thereby the effects of gene modification or changes of environmental factors on growth processes. Given the sensitivity of leaf area expansion to water deficit, monitoring and modelling leaf growth is especially important to assess the impact of unfavourable growing conditions such as droughts (Boyer 1970, Randall & Sinclair 1989).

Leaf growth of many crops and also of plant species, which are not directly linked to human needs, has been investigated for decades (Avery 1933, Boyer 1970, Walter & Schurr 1999, Wiese et al. 2007, Mielewczik et al. 2013). These investigations were conducted to study plant physiology in general but also to gain information about important agronomical plant traits. In particular, soybean plant and leaf growth has been measured and analysed by applying various quantitative methods, differing in their temporal and spatial resolution. These measurements were either done manually with the help of a ruler or similar devices (Bunce 1977, Randall et al. 1989, Ainsworth, Walter & Schurr 2005) and more recently with the help of image-based methods that can quantify leaf growth at a high temporal resolution (Ainsworth et al. 2005, Mielewczik et al. 2013). In most studies, measured leaves were chosen at a random position or the position on the plant was not even specified. So far, to our knowledge, no study has investigated diel leaf growth patterns at consecutive leaf positions on the same plant over a longer period for soybean or any other plant species. It is therefore unclear, whether diel leaf growth patterns remain constant throughout the development of a plant. Recent studies indicated that the timing of maxima and minima of leaf growth shifts during individual
leaf development of *A. thaliana* (Pantin *et al.* 2011). This shift was assigned to alterations in the sink-source relations of the plant and to a shift in the influence of hydraulic and metabolic control on leaf expansion. Therefore, it is conceivable that succeeding leaves display different rhythms of expansion.

The aim of this study was to test the hypothesis that young leaflets on the same soybean plants at different phenological stages display an equal diel growth pattern. Therefore, we measured growth from the primary leaves to leaflets of the seventh trifoliate leaf, hence, until the termination of vegetative plant growth. Soybean was taken as a model plant for this study since it has a monopodial morphology (unbranched shoot with a clear succession of leaves) and since it is a relevant major crop.

### 3.2 Materials and methods

Soybean plants (*Glycine max* (L.) Merrill, variety “Gallec”) were grown in black plastic pots (10 cm x 10 cm x 10 cm) filled with a commercial potting mix substrate (“Spezialmischung 209”, RICOTER Erdaufbereitung AG, Aarberg, Switzerland). The plants were situated in a climate chamber (Conviron, Winnipeg, Canada) under controlled conditions with a light/dark photoperiod of 13:11 h and a light intensity of 580 ± 75 μmol PAR m$^{-2}$ s$^{-1}$. Exact time points, when lights were switched on and off are given in the figures. The average temperature was 24°C during the light period and 20°C during the dark period and relative humidity was kept constant at 60%. Two types of fluorescent lamps with a 2:1 mixture were installed in the climate chamber (Master TL5 HO 54W/840, Koninklijke Philips Electronics N.V., Eindhoven, the Netherlands and FHOS4W/T5/GRO, Havells Sylvania Europe Ltd, London, UK).

For the first measurement period, young and most recently unfolded primary leaves of five plants were separately fixed in a round metal frame with strings that were glued to the leaf margin and taut using weights of around 9 g (Figs 3.1 & S1). Black plastic beads (5 mm diameter) were threaded onto the strings and also glued to the margin of the leaf to provide artificial landmarks. On top of each of these leaves, a monochrome CMOS camera (maximal resolution of 2592 × 1944 pixels) with a narrow bandpass interference infrared filter (940 nm) and a metal ring with six infrared LED clusters (940 nm) were installed.
Further details of the set-up and all technical equipment are published in Mielewczik et al. (2013). However, in contrast to that set-up, an additional round metal frame was mounted at a distance of approximately 15 cm above the frame with the fixed leaf. The strings were passed below the first and above the second frame and subsequently tautened by the weights (Figs 3.1A,B & S1B). This adaptation made it possible to keep the fixed leaf in the focal plane of the camera for a longer time than with only one metal frame, where the leaf often grew towards the camera after some time.

Digital images of these fixed primary leaves were taken every 90 seconds for six days. Next, the terminal leaflets of the second trifoliate leaves of the same five plants were measured for six days (Tab. 3.1). Terminal leaflets of the third trifoliate leaves of plant No. 1-4 and of one additional plant (Plant No. 6) of the same age and phenological stage were measured for five days. From plant No. 2-6, the terminal leaflets of the fifth trifoliate

Figure 3.1. Experimental set-up in the climate chamber. (a) Overview of the set-up in the climate chamber with five cameras. (b) Close-up view of the fixed soybean leaf with attached black beads. (c) Original image of a soybean leaf taken with an infrared camera.
leaves were measured for six days. Finally, the terminal leaflets of the sixth or seventh trifoliate leaves from plant No. 3, 4 and 6 were measured for six days. The sizes of the plants at the beginning of the different measurement periods are shown in figure S2. Due to the fact that, at the beginning of the measurement, leaflets of the third trifoliate leaves had already reached an average leaf size markedly larger than all other measured leaflets, measurements on younger leaflets of the third trifoliate leaves on six additional soybean plants were conducted in a separate set of plants at a later point in time (Fig. S3).

At the end of each measurement period, the image sequences were analysed with the software “Martrack Leaf”. This software requires a few initial user-defined steps to specify the initial locations of the beads that are delimiting the leaf area. In the first image of an image sequence, the centre of each black bead has to be selected manually. Then, a template size around the bead has to be defined and finally, a search length has to be specified which defines the neighbourhood, in which the bead has to be found in subsequent images. Once this initial procedure has been performed, the software is able to process all images and determine the position of every bead automatically. The area of the polygon defined by the bead positions is calculated for every image with the Gauss-formula for trapezoids, which results in a good approximation of the area of the actual leaf:

$$A = 0.5 \sum_{i=1}^{n} y_{i+1}x_i - y_ix_{i+1} \quad (1)$$
A is the area of the polygon, \( x \) and \( y \) are the coordinates of the \( n \) corner points of the polygon. The indices are regarded modulo \( n \), which means \( x_{n+1} = x_1 \). The relative growth rate (RGR) between two images (frames) is calculated with the following equation, where \( A_1 \) is the area at the first frame \( f_1 \), and \( A_2 \) and \( f_2 \) correspond to the second frame, respectively:

\[
\text{RGR (in \% per frame)} = \frac{(\ln A_2 - \ln A_1)}{f_2 - f_1} \times 100
\]

A time correction factor \( f_c \), corresponding to the number of images taken per hour, is needed to calculate the RGR in per cent per hour for every image:

\[
\text{RGR (in \% per hour)} = \text{RGR (\% per frame)} \times f_c
\]

In our measurements, images were acquired every 90 seconds. Hence, a time correction factor of 40 images per hour was used throughout all calculations. For each leaf, the following relative growth rates were calculated:

- **RGR (% h\(^{-1}\))**: Relative growth rate in per cent per hour calculated over a 3 h period (1.5 h before to 1.5 h after a point in time).
- **RGR (d\(^{-1}\))**: Multiplication of the above mentioned RGR with a factor of 100/24 to allow for a better comparability of our results with the results of other reports.
- **RGR (normalised)**: Relative growth rate normalised to the mean relative growth of the whole day i.e. a 24 h period ranging from 00:00 to 24:00.
- **RGR (% h\(^{-1}\); day & night mean)**: Mean relative growth rate in per cent per hour calculated separately for the light and dark period, respectively (day: from 09:30 to 19:30, night: from 22:30 to 06:30).

A detailed description of the software “Martrack Leaf” is published in Mielewczik et al. (2013). “Martrack Leaf” is provided online, compiled for different operating systems (Windows 64-bit, linux and Mac). To execute “Martrack Leaf”, the Matlab Compiler Runtime (MCR; version R2012a 64-Bit) (The Mathworks, Natick, MA, USA) needs to be installed on the user machine (download at http://www.mathworks.com/products/compiler/mcr/).
3.3 Results

The first aim of our analyses was to elucidate whether leaves on the same position situated at different plants display a similar diel growth pattern. Therefore, the diel growth patterns of five simultaneously measured primary leaves of five plants were compared (Fig. 3.2A). These leaves showed largely similar growth patterns that were characterised by three features: a) pronounced diel fluctuation of RGR b) maximal RGR towards the end of the night c) decline of RGR from the beginning to the end of the measurement. There were some deviations from these general findings: In plant 2, RGR increased from the first night to the second night and in plants 3 and 4, maximal nocturnal RGR was observed earlier in the night – especially towards the end of the measurements. At the very beginning of the night, most of the diel growth patterns (especially for plants 1, 2 and 5) showed first a slight decrease followed by a pronounced increase of RGR. During the first 48 h, RGR sometimes exceeded 1% h\(^{-1}\).

The second aim of our analyses was to compare growth between leaves on different positions. Therefore, the data from the primary leaves were averaged and normalised in a number of different ways (Fig. 3.2B-E): First of all, diel RGR of all five leaves were averaged (Fig. 3.2B). Then, the data from all individual leaves were normalised for each 24 h period (Fig. 3.2C) and this data was averaged as well (Fig. 3.2D). Finally, a temporal recalculation of the data was performed (Fig. 3.2E) in which average RGR for the entire population of five leaves for the day and for the night were calculated, respectively. These averaged and normalised data sets show similar features as the ones described above for the raw data: High nocturnal and low diurnal growth activity with a limited degree of variability between the individual leaves.

This measurement and analysis procedure was then performed for all subsequent leaves thereafter with the exception of the first and fourth trifoliate leaf, which were already developed very far when the primary leaves and the third trifoliate leaves had stopped growing, respectively. Therefore, the second trifoliate leaf was the next leaf stage to be analysed after the primary leaves (Fig. 3.3). In leaflets of the second trifoliate leaves, a maximum in RGR was first reached during the early morning hours of the second day and not during the night hours of the first night. Due to the calculation of average diurnal and
of average nocturnal growth rates, the data for the low temporal resolution (Fig. 3.3E) of the first 24 h looks markedly different, when leaflets of the second trifoliate leaves and primary leaves are compared and leaflets of the second trifoliate leaves seem to perform a pronounced shift in their development from the first 24 h interval to the second 24 h interval. For all leaves investigated here, the maximum intensity of RGR was higher than in primary leaves and reached values of almost 2% h^{-1}. The general pattern of RGR and the variability between the leaves from the five plants was very similar to the findings reported for the primary leaves.

In the third trifoliate leaf (Fig. 3.4), in the fifth trifoliate leaf (Fig. 3.5) and in the last trifoliate leaf (Fig. 3.6), variabilities between individual leaves are comparably small and the same general diel growth patterns as described above for the second trifoliate leaf can be observed. The main difference between leaves from all positions seems to reside in the first 24 h of analysis: In trifoliate leaves two, three and five, there is a tendency for higher RGR during the light period compared to the preceding night, whereas in the primary and the final trifoliate leaves, the typical diel growth pattern with higher RGR at night is visible from the beginning of the measurements on. In the additional measurements conducted on leaflets of the third trifoliate leaves maximum RGR during the first two days occurred at dawn. During the following days, the growth maximum gradually shifted towards the middle of the night (Fig. S4).

For all positions, the maximum but also the minimum RGR decreased day by day. However, the decline of the maximum RGR exceeded the one of the minimum RGR. Thus, the amplitude (difference between maximum and minimum growth rate) of the RGR continuously decreased and converged to a relatively low level at the end of the measurement period. To compare the growth rates during the same day, we calculated the relative growth rate normalised to the mean relative growth rate of the corresponding day. The maximum normalised RGR for the primary leaves and leaflets of the second to the sixth/seventh trifoliate leaves increased gradually whereas the minimum normalised RGR remained roughly on the same level (Figs 3.2C, 3.3C, 3.4C, 3.5C & 3.6C). A comparison of mean area growth rates calculated in the unit d^{-1} is shown for leaves from all positions in the supplemental data (Fig. S5). This data shows clearly the highly simultaneous growth patterns of leaves on different positions.
Figure 3.2. Diel growth pattern of soybean leaves (primary leaves). (A) Relative growth rate (RGR; % h\(^{-1}\); 3 h mean) and area growth (1 d\(^{-1}\); 3 h mean) of five leaves. (B) Mean RGR (% h\(^{-1}\) ± SE; 3 h mean) and area growth (1 d\(^{-1}\) ± SE; 3 h mean) (n = 5). (C) RGR of five leaves normalised to the 24 h period. (D) Mean RGR (normalised ± SE; n = 5). (E) Mean RGR (% h\(^{-1}\) ± SE; mean for day and night; n = 5).
Figure 3.3. Diel growth pattern of soybean leaves (second trifoliate leaves). (A) Relative growth rate (RGR; \% h\(^{-1}\); 3 h mean) and area growth (1 d\(^{-1}\); 3 h mean) of five leaves. (B) Mean RGR (\% h\(^{-1}\) ± SE; 3 h mean) and area growth (1 d\(^{-1}\) ± SE; 3 h mean) (n = 5). (C) RGR of five leaves normalised to the 24 h period. (D) Mean RGR (normalised ± SE; n = 5). (E) Mean RGR (\% h\(^{-1}\) ± SE; mean for day and night; n = 5).
Figure 3.4. Diel growth pattern of soybean leaves (third trifoliate leaves). (A) Relative growth rate (RGR; % h⁻¹; 3 h mean) and area growth (1 d⁻¹; 3 h mean) of five leaves. (B) Mean RGR (% h⁻¹ ± SE; 3 h mean) and area growth (1 d⁻¹ ± SE; 3 h mean) (n = 5). (C) RGR of five leaves normalised to the 24 h period. (D) Mean RGR (normalised ± SE; n = 5). (E) Mean RGR (% h⁻¹ ± SE; mean for day and night; n = 5).
Figure 3.5. Diel growth pattern of soybean leaves (fifth trifoliate leaves). (A) Relative growth rate (RGR; % h⁻¹; 3 h mean) and area growth (1 d⁻¹; 3 h mean) of five leaves. (B) Mean RGR (% h⁻¹ ± SE; 3 h mean) and area growth (1 d⁻¹ ± SE; 3 h mean) (n = 5). (C) RGR of five leaves normalised to the 24 h period. (D) Mean RGR (normalised ± SE; n = 5). (E) Mean RGR (% h⁻¹ ± SE; mean for day and night; n = 5).
Figure 3.6. Diel growth pattern of soybean leaves (sixth/seventh trifoliate leaves). (A) Relative growth rate (RGR; % h\(^{-1}\); 3 h mean) and area growth (1 d\(^{-1}\); 3 h mean) of three leaves. (B) Mean RGR (% h\(^{-1}\) ± SE; 3 h mean) and area growth (1 d\(^{-1}\) ± SE; 3 h mean) (n = 3). (C) RGR of three leaves normalised to the 24 h period. (D) Mean RGR (normalised ± SE; n = 3). (E) Mean RGR (% h\(^{-1}\) ± SE; mean for day and night; n = 3).
3.4 Discussion

Leaf growth measurements in our study were conducted in a climate chamber. This allowed us to keep environmental conditions constant throughout the experiments and thereby, to accurately examine, whether young leaflets of the same soybean plant display a similar diel growth pattern at different phenological stages. Due to the user-friendliness of the growth imaging method (Mielewczik et al. 2013), it was possible to analyse a high number of leaves in a limited time. This was an important prerequisite in order to test the hypothesis that diel growth patterns of successive leaves are comparable. As shown in figures 3.2 to 3.6, this hypothesis was confirmed and highly consistent diel growth patterns were found for leaves at different positions on the plant. This is not self-evident, since it is well known that leaves at different positions grow with different intensities and reach different final sizes, which depend on the environment (Walter, Roggatz & Schurr 2003).

The maximum growth rate shown by most examined leaves was found approximately in the middle of the night. This is in accordance with findings shown in Ainsworth et al. (2005) where the maximum growth of soybean leaves also occurred in the middle of the night. In that study, the peak of the maximum RGR was more obvious in the controlled conditions of a growth chamber (L:D 12:12 h) compared to less tightly controlled conditions in the greenhouse (L:D 15:9 h). Bunce (1977) also found that the leaf elongation rate of soybean is greater at night, and Boyer (1968) reported that the leaf growth for sunflower is higher at night. Walter, Silk & Schurr (2009) suggested that for dicot species there are two major types of diel leaf growth cycles. The first type shows the maximum growth rate during early morning hours. Among others, *Ricinus communis, Nicotiana tabacum* and *Arabidopsis thaliana* belong to this type. Plants belonging to the second type show their maximum growth rate at the end of the day or in the early night. This growth pattern has been observed for leaves of *Populus deltoides, Chamaecyparis sp.* and *Clusia minor*. Also soybean was suggested to fall into this class, based on the observation of a maximum growth during the middle of the night, which seems less clear now based on the result of this study.
Apart from similarity of the overall diel growth patterns, leaves at all measured positions on the plant also showed similar short-term growth fluctuations. Soon after the light was turned off, the leaves first displayed a short decrease in their RGR followed by a prolonged increase. This phenomenon was reported already in earlier studies for single leaves of several plant species (Ricinus communis and Nicotiana tabacum: Poiré et al. 2010, N. tabacum: Walter 2009, Walter & Schurr 2005, A. thaliana: Wiese et al. 2007) and it is now shown that this phenomenon occurs in leaves of all positions within the plant. The amplitudes of the growth rate for the leaflets of the second to the fifth trifoliate leaves were in a similar range over their measurement periods. However, the amplitudes for the primary leaves and the leaflets of the sixth/seventh trifoliate leaves were lower. This is caused by the fact that primary and sixth/seventh leaves reached smaller final leaf sizes compared to the second to fifth leaves. Therefore, at the onset of analyses, they were already closer to the full grown stage.

Randall & Sinclair (1989) found that the RGR of leaves which had reached more than 20% of their final leaf area was significantly lower in a drought-stressed treatment than in an irrigated treatment. For leaves that had reached less than 20% of their final leaf area, no such difference was observed. This strongly indicates that the expansion process in small leaves is relatively insensitive to drought stress and that a leaf-growth based monitoring of drought stress will most beneficially be possible in large leaves.

In our study, most of the investigated leaves were already larger than 20% of their final leaf area at the beginning of the measurement. Therefore, in future drought stress experiments our approach should be suitable to discriminate between genotypes that suffer severely from drought stress and genotypes that can better cope with such a situation.

For leaflets of the second, third and fifth trifoliate leaf, the maximum growth rate observed during the first and second day after the fixation of the leaf was reached a few hours later – at dawn or after the onset of light – than observed for the primary leaves and leaflets of the sixth/seventh trifoliate leaves. This could either be an indication that a very young leaf needs some time to adjust to the mechanically restricted position or an indication for a slight developmental shift of the diel leaf growth pattern. As indicated
above, primary leaves and leaflets of the sixth/seventh trifoliate leaves reached a smaller final leaf size than all other leaves measured. Thus, when these leaves unfolded and the measurement started, they had reached more than 40% of their final leaf size and probably already shifted their growth pattern. Measurements in a markedly earlier developmental stage of primary leaves or of the sixth/seventh trifoliate leaves would not have been possible, since unfolding in leaves of all positions is completed only when leaves reach a size of three to four cm\(^2\), corresponding to 10% of the final leaf size in leaves of middle positions and to often more than 30% in earliest or latest leaves of soybean.

Mechanical restriction preventing nyctinastic leaf movements is known to affect carbohydrate metabolism thereby leading to somewhat diminished growth in castor bean leaves (Walter, Feil & Schurr 2002). This reaction starts immediately after the onset of tensile forces and it might be gradually relieved. It is conceivable that it is only visible in leaves which grow intensely and not in leaves that reach a smaller final leaf size (such as the primary leaf and leaflets of the sixth/seventh trifoliate leaf). The close connection between carbon metabolism and diel leaf growth pattern is well known (Kehr et al. 1998, Walter et al. 2009) and needs to be investigated in more detail in future studies. At the moment, it cannot be excluded that carbohydrate metabolism has been affected during the adjustment period, when tensile forces are beginning to affect leaf growth.

A slight developmental shift in the phasing of the diel leaf growth pattern is conceivable as well and this would also be related to carbohydrate metabolism as pointed out recently by Pantin et al. (2011). They showed that timing of minima and maxima of leaf growth shifted markedly during individual leaf development in A. thaliana. This shift was assigned to alterations in the sink-source relations of the plant and to a shift in the influence of hydraulic and metabolic control on leaf expansion. In their study, leaf growth was analysed only in leaves from a single position, leaving open the question, whether a general shift of sink-source-relations during plant development would lead to different diel growth patterns in leaves from different positions. Moreover, Pantin et al. (2011) computed diel leaf growth patterns based on merely three images per day and recalculated the measured growth rates to a reference temperature of 20°C. Relative expansion rates (RER) for the light and the dark period, respectively, were given. Thus, this shift of the RER might have been prone to over-interpretation due to the low temporal
resolution and the recalculation to a reference temperature, for which the empirical basis is unclear. Yet, the occurrence of a developmental shift of the phasing of the diel leaf growth pattern in very rapidly growing leaves would be consistent with our results as well and this question should be elucidated in more detail in various species in future studies.

Another important set of questions that can be elucidated on the basis of our results is how changing environmental conditions and/or stress situations (such as drought) affect leaf growth. Such studies would help identifying genotypes that can better cope with unfavourable growing conditions such as droughts or they could clarify potentially beneficial alterations in the diel growth patterns of genetically modified plants as reported in Preuss et al. (2012).

3.5 Conclusion

In our study, all measured leaves/leaflets showed a consistent diel growth pattern. In particular, the leaflets of the third to the fifth trifoliate leaves experienced nearly equal growth amplitudes. Hence, we conclude that monitoring the stress level of a plant via the RGR can be conducted on any leaflet of a soybean plant. Some care needs to be taken in intensely growing leaves at very early developmental stages. Here, either the mechanical fixation required for this analysis method or a developmental shift in the carbohydrate metabolism might lead to slightly altered phasing of the diel growth rhythm. Yet, overall, all leaves within the canopy show very consistent diel growth patterns.

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3.6 References


Chapter IV

Diel leaf growth pattern of soybean (*Glycine max*) under simulated field conditions: night temperature matters!

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Abstract

Leaf growth, gene expression and plant metabolism are tightly controlled by the circadian clock – in a way, which optimizes the plant’s performance to prevailing environmental conditions. Usually, climate chamber experiments with constant nighttime and daytime temperature regimes are performed to elucidate plant performance and regulatory mechanisms. A recently developed imaging approach allowed to analyze the leaf growth of soybean at high temporal resolution under field conditions. Results from these measurements revealed coinciding patterns of growth rate and air temperature with most rapid growth in the afternoon. This is in contrast to earlier results on the timing of leaf growth of dicot plants.

In this study, we modified temperature regimes in climate chambers in a way that mimicked field conditions as good as possible. Under these conditions, a diel leaf growth pattern very similar to that observed in the field occurred. This pattern reverted to a pattern previously described in literature with maximum growth in the early morning, when temperature conditions were reverted to standard climate chamber settings within two days. As shown by comparison with analyses for wheat leaf growth and based on a large body of literature, the relation between temperature and growth – especially at night – seems to be very different for typical dicotyledonous plants and grasses. The results of this study point on the enormous importance of nighttime temperature not only for leaf growth, but most probably for underlying control processes of metabolism and gene regulation.
4.1 Introduction

Leaf growth, gene expression and plant metabolism are tightly controlled by internal oscillators called the “circadian clock” and externally by the environment (McClung 2001, Nozue et al. 2007, Farré 2012, Ruts et al. 2012). Plants use the circadian clock to anticipate the daily but also the seasonal fluctuations in their environments (Ruts et al. 2012).

Leaf growth reacts very sensitively towards changing environmental conditions. Temperature and other environmental parameters can affect leaf growth and potentially even alter the intrinsic diel (24 h) leaf growth pattern. Therefore, short-term leaf growth analyses allow for the assessment of the tolerance of a plant to abiotic stress such as drought (Banziger et al. 1999), heat (Lipiec et al. 2013) or nutrient deficiency (Walter et al. 2009). Leaf growth of a wide range of plant species has been investigated since the last century (e.g. Avery 1933, Boyer 1970, Walter & Schurr 1999). In monocotyledonous, graminoid species, a linear correlation between temperature regime and diel leaf extension was observed (Peacock 1975, Gallagher & Biscoe 1979, Gallagher et al. 1979, Sadok et al. 2007). Only very recently, experiments in maize pointed out that also for graminoid species, intrinsic growth patterns exist that are connected to the circadian clock (Caldeira et al. 2014). In broad-leaved, dicotyledonous species, clear signs of circadian rhythms were found from early measurements on, which showed only very limited correlation between temperature regime and diel leaf growth variation (Bunce 1977, Walter et al. 2009, Poiré et al. 2010b). Early measurements in dicot species were performed manually with the help of a ruler or similar devices and thus temporal resolution was limited (Bunce 1977, Randall & Sinclair 1989). Many studies were carried out to gain information about important agronomical plant traits but partially also to study plant physiology. However, often studies have been conducted under controlled conditions such as in climate chambers or greenhouses. Hence, still today, it is often unclear to which extent findings achieved under controlled conditions can predict the performance of a plant in the field.

In more recent approaches, leaf growth of dicot species was measured at high temporal resolution with the help of imaging methods (e.g. Schmudt et al. 1998, Ainsworth et al. 2005, Poiré et al. 2010a). In a so-called digital image sequence processing (DISP) approach,
leaf growth was measured two-dimensionally by analyzing an image sequence of a growing leaf (Schmundt et al. 1998). However, because of the high sensitivity to fluctuating illumination conditions, this method can hardly be used outside of climate chambers providing constant illumination. Therefore, diel leaf growth analyses of dicot plants were usually not conducted in field experiments.

Only recently, an imaging approach was developed that allows the analysis of two-dimensional leaf growth at high temporal resolution under field conditions (Mielewczik et al. 2013). We applied this approach to analyze the leaf growth of soybean as a model plant in a field in Switzerland from 2012 onward. From growth chamber studies, it is well known that soybean shows a pronounced peak of diel leaf growth activity towards the end of the night or at the beginning of the day (Ainsworth et al. 2005, Friedli & Walter 2015). A few properties of the setup described in Mielewczik et al. (2013) had to be adapted to perform the measurements in the field in a reliable manner, resulting in an intense measurement campaign in 2014.

Based on these field results, we tested the hypotheses, whether under controlled conditions of a climate chamber (1) the peak of diel leaf growth activity is realized during the light period if plants are grown under cold temperatures during the dark period and warm temperatures during the light period; (2) the peak of leaf growth reverts to the dark period, if plants acclimated to cold temperatures during the dark period are transferred to warm temperatures during the dark period. In addition, we monitored diel leaf extension of wheat as a graminoid species in the field and in climate chamber conditions under warm and cold temperature regimes to verify whether the established findings of literature hold true also under the conditions which we were able to apply to our plants that originate from Swiss breeding programs and that are commercially used in Switzerland and neighboring countries in the field.
4.2 Results

4.2.1 Leaf elongation growth of wheat

Leaf elongation growth of wheat in the climate chamber showed a remarkable coincidence between patterns of growth rate and air temperature throughout the measurement periods (Figure 4.1A+B). Both under warm (Figure 4.1A) and cold temperature regimes (Figure 4.1B) coinciding patterns of growth rate and air temperature could be observed. As soon as the temperature started to increase in the morning, the growth rate increased likewise in a relatively parallel manner to temperature until the maximum temperature was reached. As soon as the temperature started to decrease, the growth rate also decreased in a coinciding manner. This observation was obtained also under unusually high night temperatures that would not be reached at this developmental stage of the plant in the field. In the field, coinciding time courses of growth rate and air temperature were observed as well (Figure 4.1C+D). However, during measurements in April, the coincidence of growth rate and air temperature was more pronounced at the beginning of the measurement period with warmer temperatures (Figure 4.1C). Towards the end of this period it was colder and the time courses of growth rate and air temperature were similar but less coinciding as observed during higher temperatures. Somewhat less coinciding time courses of growth rate and air temperature were also observed during the measurement period in May (Figure 4.1D).

4.2.2 Leaf area growth of soybean

Leaf growth of soybean measured in the field was more intense during the day than during the night (Figure 4.2A). At the beginning of the day, the relative growth rate (RGR) increased until the afternoon and then started to decline again. During the night, the growth rate stayed relatively constant at around 0.5% h\(^{-1}\) until the next morning when the growth rate again strongly increased. The observed growth pattern of soybean in the field is in contrast to the literature, where it is reported that the diel leaf growth pattern of soybean shows a maximum growth towards the end of the night (Figure 4.2B; Ainsworth et al. 2005, Friedli & Walter 2015).
Figure 4.1. Leaf elongation growth of *T. aestivum*. Measurements conducted in the climate chamber under warm climate conditions (A; n=30) and cold climate conditions (B; n=20). Measurements conducted in the field: 24.-30.04.2013 (C; n=14); 01.-05.05.2013 (D; n=6). Shaded areas indicate the dark period in the climate chamber and the period between sunset and sunrise in the field, respectively. LER = leaf elongation rate.

Figure 4.2. Mean diel leaf growth pattern of *G. max* in the field (A; n=12; 3h mean) and in a climate chamber with “conventional” temperature conditions (B; n=6; 3h mean). Shaded areas indicate the period between sunset and sunrise in the field and the dark period in the climate chamber, respectively. Climate chamber data adapted from figure S4 of Friedli & Walter 2015 with kind permission of Wiley. RGR = relative growth rate.
The aim of experiment I was therefore to elucidate whether leaf growth of soybean occurs mainly during the light period, if plants are grown under cold temperatures during the dark period and warm temperatures during the light period as it was observed by leaf growth measurements on soybean in the field (Figure 4.2A). In experiment I, main growth of young leaves (terminal leaflets of trifoliate leaves (TL)) occurred during the light period (Figure 4.3). Directly after the light was turned on, the RGR of all leaves decreased and increased sinusoidally. The same effect was observed at the end of the light period. The difference between growth during the light period and during the dark period became less obvious with increased leaf areas (LAs). Until a relative leaf area (RLA) of 70% was reached, the difference between growth during the light period and during the dark period was highly significant or significant (Table 4.1).

Figure 4.3. Mean relative growth rate of G. max in experiment I. Leaves are grouped by their leaf area relative to fully grown leaves. Shaded areas indicate the dark period.

Table 4.1. Difference between the relative growth rate (RGR; % h⁻¹) of G. max during the light period (06:00-23:00) and the dark period (23:00-06:00) in experiment I. Leaves are grouped by their leaf area relative to fully grown leaves. P-values are based on a one-sided Welch’s t-test. Only groups with n > 5 are shown.

<table>
<thead>
<tr>
<th>Relative leaf area (%)</th>
<th>RGR day (% h⁻¹)</th>
<th>RGR night (% h⁻¹)</th>
<th>P-value</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-30</td>
<td>1.13</td>
<td>0.36</td>
<td>&lt; 0.001</td>
<td>18</td>
</tr>
<tr>
<td>30-40</td>
<td>0.96</td>
<td>0.42</td>
<td>&lt; 0.001</td>
<td>34</td>
</tr>
<tr>
<td>40-50</td>
<td>0.89</td>
<td>0.42</td>
<td>&lt; 0.001</td>
<td>29</td>
</tr>
<tr>
<td>50-60</td>
<td>0.71</td>
<td>0.41</td>
<td>&lt; 0.001</td>
<td>25</td>
</tr>
<tr>
<td>60-70</td>
<td>0.65</td>
<td>0.41</td>
<td>0.004</td>
<td>18</td>
</tr>
<tr>
<td>70-80</td>
<td>0.56</td>
<td>0.35</td>
<td>0.067</td>
<td>8</td>
</tr>
</tbody>
</table>
For higher RLAs the difference was not significant. Leaf growth during the light period decreased with increased RLA on all measured leaf positions (Figure 4.4A) but remained constant during the dark period (Figure 4.4B). By comparing the mean growth rate of the light period with the RLA, a high correlation (Spearman-correlation: $r_s = -0.867$) was obtained. The growth rate during the light period strongly correlated with temperature for RLAs of 20-80% ($R^2 = 0.920$ to 0.972) between 09:15 and 21:00 (Figure 4.5). Leaves of higher position on a plant reached larger final LAs than leaves of lower position (Table 4.2).

Figure 4.4. Correlation between relative leaf area and relative growth rate of G. max in experiment I during the light period (A) and the dark period (B). Data are shown for trifoliate leaves (TL) 1 to 4.

Table 4.2. Mean leaf area (mm$^2$ ± SE) of terminal leaflets of G. max 93 days after sowing.

<table>
<thead>
<tr>
<th>Trifoliate leaf nr.</th>
<th>Mean area (mm$^2$ ± SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1860 ± 94</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>2530 ± 137</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>3065 ± 127</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>3028 ± 148</td>
<td>11</td>
</tr>
</tbody>
</table>
Diel leaf growth pattern of soybean under simulated field conditions

Figure 4.5. Correlation between temperature and relative growth rate (RGR; % h⁻¹) of *G. max* in experiment I. Leaves are grouped by their leaf area relative to fully grown leaves: 20-40 % (A; n=36); 40-60 % (B; n=32); 60-80 % (C; n=12). Shown are the data of the period between 09:15 and 21:00.

The aim of experiment II was then to elucidate, whether the peak of growth activity reverts to the dark period, if plants acclimated to cold temperatures during the dark period are transferred to warm temperatures during the dark period. Already during the dark period between day 2 and day 3 in which the temperature was increased to warmer temperatures, an increased growth rate, compared to the previous dark period, was achieved (Figure 4.6 and table S4.1 for more details). In the following two days, the growth rate still showed a growth peak during the light period, although lower than observed in experiment I. From day 5 of experiment II onwards, this growth peak during the light period disappeared. By comparing the mean growth rate during the light and dark period, it became apparent that after the switch of the climate conditions, there was an immediate shift of growth activity from the light period to the dark period (Figure 4.7). By comparing the mean growth rate during the light period and during the dark period, respectively, of experiment I with the mean growth rates obtained in experiment II, it became evident that most pronounced growth differences between the two experiments were obtained at night immediately after the shift in temperature regime (Figure S4.2).
Figure 4.6. Diel growth pattern of *G. max* in experiment II. (A) Mean relative growth rate (% h\(^{-1}\) ± SE) of TL 2 (red line), TL 3 (blue line) and TL 4 (green line); n=6 per TL; (B) Temperature (°C) and (C) relative humidity (%) conditions during the experiment. Shaded areas indicate the dark period.

Figure 4.7. Mean relative growth rate of *G. max* during the light period and the dark period in experiment II. The blue lines show the mean growth rate during the light period (08:00-21:00) and the red line the growth rate during the dark period (21:00-08:00) of TL 2 (A), TL 3 (B) and TL 4 (C) (n=6 per TL). The green line shows the point in time when the climate settings were switched from the simulated to the “conventional” conditions.
4.3 Discussion

The results of this study mainly showed two interesting aspects: a) The pronounced temporal shift of maximal soybean leaf growth activity as a response to small changes in temperature regimes b) constant nocturnal soybean leaf growth activity under low temperature throughout an extended period of leaf development. Such phenomena were not observed in wheat and these phenomena have not been reported for diel leaf growth patterns of dicot plant species before.

Leaf growth measurements on soybean in the field revealed most rapid growth in the afternoon and coinciding time courses of growth rate and air temperature (Figure 4.2A). This finding is in contrast to results from the literature (Bunce 1977, Ainsworth et al. 2005, Friedli et al. 2015). Moreover, the diel growth pattern changed rapidly, when temperature settings were switched to more usual climate chamber settings. This finding clearly reveals that leaf growth and growth-controlling factors strongly depend on the exact temperature regime. Deviations from naturally occurring temperature regimes can obviously lead to growth peaks at unusual times such as in the night or early morning. When growth is maximal at such times, e.g. growth-related carbohydrate metabolism, turgor pressure and leaf hydraulics need to be adjusted accordingly. It is well known that carbohydrate metabolism and growth are closely coupled (Pantin et al. 2011, Stitt & Zeeman 2012) and that carbohydrate metabolism is closely controlled by the circadian clock (Graf et al. 2010, Farre & Weise 2012). Therefore the question how aberrant diel growth patterns feed back into the plant’s demand for carbohydrates and into the interaction of physiology with the circadian clock needs to be closely studied in future research. There is a certain probability that climate chamber based studies relying on warm night temperature or constant 24 h temperatures suffer from a growth-dependent carbohydrate demand which occurs at a very unusual time during the diel cycle. Other than air temperature, also the temperature of the root zone can influence the diel leaf growth pattern (Poiré et al. 2010a). Since root-zone temperature in turn depends on radiative heating of the dark pots used in climate chamber based experiments, another level of complexity might affect the relation between climate factors and leaf growth in non-field experiments.
As shown by the analyses of wheat leaf elongation (Fig. 4.1), the relation between leaf growth and temperature seems to be much more straightforward in graminoids compared to soybean and putatively to other dicotyledonous species. In wheat, temperature and leaf elongation rate develop in parallel, sometimes with a slight temporal shift (Fig. 4.1). This points to the well-known linear relations between temperature and development that hold true for a relatively wide range of temperatures, also when the exact timing of the temperature regime applied in a growth chamber is not simulating temperature regimes that would be expected in the field at this developmental stage of the plant (Peacock 1975, Gallagher & Biscoe 1979, Gallagher et al. 1979, Sadok et al. 2007).

In contrast to this, in our experiment I with soybean, a linear correlation between leaf growth and temperature was observed only (Fig. 4.5) when the temperature regime mimicked the temperature regime of the field conditions. With a somewhat artificial temperature regime, the parallelism between growth and temperature is lost rapidly: The diurnal growth rates of between 1 and 0.5% h\(^{-1}\) reached between day 4 and day 6 of experiment II at a temperature of 26°C (Fig. 4.6) would still be in line with the correlations reported in Fig. 4.5. Yet, the nocturnal peak growth rates of 1.5% h\(^{-1}\) and more at a temperature of 20°C are far out of the range of correlations displayed in Fig. 4.5. This points to the existence of a strong intrinsic diel growth cycle that has a maximum at the expected time of maximal temperatures during the day. If night temperatures exceed expected values, the system is disturbed and reacts in a nonlinear way with high growth activity that needs to deplete carbohydrate reserves to provide the necessary material for the increase of tissue such as carbohydrates for cellulose of the cell wall. Since also under natural climate conditions in the field, a high variability of night temperatures can occur, it will be interesting in future studies to see a) how fluctuations in field temperature conditions affect diel leaf growth patterns there and b) whether there are growth differences e.g. between more and less cold-tolerant genotypes at different night temperatures. Huxley et al. (1976) showed that the growth and yield components of soybean are strongly dependent on the temperature during the night. It is conceivable that this points to a genotypic variability for the depletion of storage compounds and in sink-source relations. Therefore, diel leaf growth analyses could be useful in breeding
programs for the selection of soybean genotypes that can achieve a more stable yield under variable climate conditions.

Another interesting observation of this study is the constant growth during the dark period (Figure 4.4B), in experiment I, which has not been reported or observed before in similar leaf growth analyses under conventional climate chamber settings. It is conceivable that leaves would have had an even lower growth rate at night under cold conditions of experiment I, but that the expansion was enforced by the constant tensile forces acting on the leaf throughout the diel cycle. For *R. communis* it was shown under ‘conventional climate chamber conditions’ that moderate tensile forces acting on a leaf with simultaneous prevention of leaf movements do not affect the intensity and temporal distribution of the overall growth rate compared to freely growing leaves (Walter et al. 2002). In other words, the diel leaf growth pattern of *R. communis* was not affected by the attached weights as long as their magnitude was chosen appropriately. However, the diurnal fluctuations in the starch content of almost fully grown leaves were affected by the tensile forces also in the case of ‘undisturbing magnitude’ of the force. Therefore, if the growth-related carbohydrate or storage-compound turnover in soybean leaves were affected strongly by nocturnal temperature, it is conceivable that the constant tensile forces can induce a comparable nocturnal growth of younger and older leaves, which might be masked in ‘awkward’ temperature regimes (that are usually applied in normal climate chamber experiments) by the above-mentioned, nonlinear effect of nocturnal growth increase.

Another interesting observation of these experiments is the transient fluctuation of growth activity at the beginning of the day and at the beginning of the night. This observation was already reported in previous studies for soybean (Friedli et al. 2015) and for other plant species (*Nicotiana tabacum*: Walter & Schurr 2005b, *Arabidopsis thaliana*: Wiese et al. 2007, Walter et al. 2009, *Ricinus communis* and *Nicotiana tabacum*: Poiré et al. 2010b). Observations with a closed-circuit television (CCTV) camera on a freely growing soybean plant revealed a rising of the leaves at the beginning of the light period and a lowering of the leaves at the end of the dark period (Figure S4.3). Thus, the forces connected to the rising (Figure S4.3A-C) and lowering (Figure S4.3G-I) of the leaves could possibly contribute to the observed transient fluctuations by minor distortions of the
analyzed leaves that are more or less tightly fixed in the focal plane of the camera. During the remainder of the light period (Figure S4.3D-F) and the dark period (Figure S4.3J-L) no remarkable leaf movements and also no bigger short-term fluctuations in the growth rate were observed.

In a review, Walter et al. (2009) reported that the diel growth pattern of monocot plants follows temperature with the highest growth rate during the day, whereas the diel growth pattern of dicot species show their maximal growth activity in the beginning of the day (type 1) or at the end of the day (type 2), irrespective or even reverse to the temperature profile. This difference between diel leaf growth patterns of monocot and dicot species is reasoned mainly by their different organization and placement of growth zones (Ruts et al. 2012). By looking in more detail on the climate conditions of the studies cited in Walter et al. (2009) that were measuring growth of dicot species, it became apparent that none of the studies applied lower temperatures than 19 °C during the dark period (Table 4.3). The differences between temperatures used during the light period and the dark period were in the small range of 0 to 6 °C. All studies, except two, reported that the main growth for the investigated species occurred during the dark period. These two studies (Walter et al. 2005a, Matsubara et al. 2006) had the biggest temperature differences between the dark period and the light period and at the same time the lowest temperatures during the dark period. In both studies, the main growth of Populus deltoides was observed towards the end of the light period. These facts support our argumentation on the importance of nighttime temperature and temperature regimes on the diel growth pattern.

As pointed out already above, leaf elongation measurements of wheat conducted in our study confirmed the knowledge of the literature about the growth of monocot species: the diel growth pattern of wheat followed the temperature under various climate conditions in the field as well as in a climate chamber (Figure 4.1). The coinciding patterns of growth by monocot and temperature without endogenous rhythmic growth, however, was doubted in a newer study. Caldeira et al. (2014) investigated whether day/night alternations of plant water status during an entrainment period induce an endogenous rhythm of maize leaf growth. They could show for maize that the amplitude of rhythmic growth under continuous light depends on the severity of temporary water stress during an entrainment period. This finding is in contrast to literature (e.g. Poiré et al. 2010b)
where no such rhythmic growth was observed for maize and rice under continuous light. Caldeira et al. (2014) argue that no such circadian rhythmic growth was observed so far in maize and also in rice due to a low evaporative demand during entrainment periods in standard climate chambers. Perhaps, the finding by Caldeira et al. (2014) initiated a new phase of research in monocot species paying more attention to climate conditions used for investigations under controlled conditions and putting a stronger focus on the elucidation of the intrinsic rhythmicity of leaf growth there. In a similar way, the observations reported in our study might show that for dicot leaf growth, the intrinsic diel growth rhythms are masked to a much stronger, highly non-intuitive extent as conceived so far by the temperature regime to which plants are exposed.

### Table 4.3. Climate conditions of studies measuring leaf growth of dicot species at high temporal resolution.

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature (°C; day/night)</th>
<th>Growth peak</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>23/20</td>
<td>early morning</td>
<td>Wiese et al. (2007)</td>
</tr>
<tr>
<td>Glycine max</td>
<td>28/28</td>
<td>night</td>
<td>Ainsworth et al. (2005)</td>
</tr>
<tr>
<td>Glycine max</td>
<td>24/20</td>
<td>night</td>
<td>Friedli &amp; Walter (2015)</td>
</tr>
<tr>
<td>Populus deltoides</td>
<td>25/19</td>
<td>early evening&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Matsubara et al. (2006)</td>
</tr>
<tr>
<td>Populus deltoides</td>
<td>35/20&lt;sup&gt;2&lt;/sup&gt;</td>
<td>early evening&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Walter et al. (2005)</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>24/24</td>
<td>early morning</td>
<td>Schmoldt et al. (1998)</td>
</tr>
<tr>
<td>Ricinus communis</td>
<td>25/25</td>
<td>early morning</td>
<td>Walter et al. (2002)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Temperature gradually increased in the morning and decreased in the evening over several hours, respectively

<sup>2</sup>Growth minimum in the early morning, continuous growth increase during the day

### 4.4 Conclusions

In our study, the effect of different temperature regimes on the diel growth pattern of soybean leaves could be revealed by leaf growth measurements under controlled conditions. The diel leaf growth pattern of soybean was changed drastically by only small modifications of the temperature regime. Thus, such a modified temperature regime most probably also affects underlying control processes of metabolism and gene expression. Usually, experiments to elucidate plant performance and regulatory mechanisms under controlled conditions are performed with very simple temperature regimes or even more with constant temperature regimes during light period and dark period. Therefore, findings achieved so far under controlled conditions may have to be reconsidered. In future studies, it is very important to use more realistic climate conditions for experiments conducted under controlled conditions that aim to predict the performance of plants in the field. Temperature regimes and especially night
temperatures need to be adjusted more precisely to better understand their effect on plant growth, metabolism and gene expression. Our results may point out an important way how to improve bridging the gap between lab and field research in plant science: Temperature regimes and especially night temperature needs to be considered more carefully in future studies.
4.5 Materials and methods

4.5.1 Leaf area growth of soybean

4.5.1.1 Experimental setup in the field

Measurements were conducted in the field of the research station for plant science of ETH Zurich in Eschikon, Lindau (Switzerland) in 2014 on leaves of soybean [Glycine max (L.) Merrill, variety ‘Gallec’]. The setup for the leaf growth measurements was arranged as described in detail in Mielewczik et al. (2013) and Friedli & Walter (2015) with the exception of two modifications. First, white beads on a black background were used instead of black beads on a white background. By the exchange of the colors of the beads and the background, disturbing shades almost never occurred which resulted in a markedly improved tracking rate. Second, weatherproof closed-circuit television (CCTV) cameras (Lupusnet HD - LE934, CMOS sensor, maximal resolution of 1920 × 1080 pixels, Lupus-Electronics® GmbH, Germany) were used to take images in the field (Figure 4.8D+E). These cameras have an internal infrared lighting, enabling to take images also during the night. Images are saved automatically on an exchangeable micro SD card. Thus, no computer was needed in the field for image storage as it was necessary for the measurements reported in Mielewczik et al. (2013) and measurements could also be conducted during rainy periods.
Figure 4.8. Experimental setup to measure leaf area growth of *G. max* in the climate chamber and in the field. (A) Overview of the setup in the climate chamber with six cameras. (B) Close-up view of the fixed soybean leaf with attached white beads in the climate chamber. (C) Original image of a soybean leaf taken with a CMOS camera. The five red lines are overlaid to the first image of an image sequence and show the path of the tracked center of each white bead over the entire sequence. The green polygon shows the area at the beginning of the sequence and the yellow polygon shows the area at the end of the sequence. (D) Overview of the setup in the field with a closed-circuit television (CCTV) camera. (E) Close-up view of the fixed soybean leaf with attached white beads in the field.
4.5.1.2 Plant cultivation and experimental setup in the climate chamber

Soybean plants [Glycine max (L.) Merrill, variety ‘Gallec’] were inoculated with ‘HiStick® Soybean Inoculant’ (Becker Underwood Limited, UK) and grown in QuickPot™ trays (88 cm³ per seedling, Herkuplast Kubern GmbH, Germany) filled with a sterilized substrate (Substrat 1, Klasmann-Deilmann GmbH, Germany) that was autoclaved at 121 °C for 30 minutes prior to sowing. After 15 days (18 days in experiment II), seedlings were transferred to clay pots (12 cm in diameter) filled with a mixture by weight of 2/3 ‘sterile Landerde’ (RICOTER Erdaufbereitung AG, Switzerland) and 1/3 fire-dried quartz sand (0.7-1.2 mm, Carlo Bernasconi AG, Switzerland). Until leaf growth measurements, plants were grown in another climate chamber (hereinafter referred to as “chamber 2”) (Conviron, Winnipeg, Canada) of the same device type and with the same climate settings as used in the climate chamber where leaf growth measurements were conducted (hereinafter referred to as “chamber 1”). Plants were watered three times per week and new plants were grown for each experiment.

In experiment I, leaf growth of soybean was measured in a climate chamber in which the climate parameters (temperature, relative air humidity, temporal cycle of light intensity) were set to the average climate conditions (see figure 4.9B+D and table S4.4 for more details) of six days (21.-26. June 2014) recorded during leaf growth measurements in the field in 2014. In these settings, the temperature changed from 26 °C during the light period to 15-12 °C during the dark period. Relative humidity (RH) was 60% during the light period and 80% during the dark period and a light/dark photoperiod of 15.3:8.7 h was used. In this experiment, plants were transferred to “chamber 1” latest six days before growth measurements started. The leaf area growth of terminal leaflets of the first to the fourth trifoliate leaf (TL1 - TL4) was measured simultaneously on six plants for around one week (Figure 4.8A). The areas of the fully grown terminal leaflets (n=9-11 per trifoliate leaf) were manually measured 93 days after sowing (DAS) (Table 4.2). The values of the fully
grown leaflets were used to calculate the relative leaf area (RLA) of the leaflets during the measurement.

![Figure 4.9](image)

**Figure 4.9.** Temperature and humidity settings of the “conventional” climate conditions (A, C) and the simulated field conditions (B, D). Shaded areas indicate the dark period.

In experiment II, the climate chamber settings were the same as describe above for experiment I for the first 2.5 days of the leaf growth measurements. Then, the climate parameters were changed to the same settings used in Friedli & Walter (2015) and kept until the end of the experiment (see figure 4.9A+C and table S4.1 for more details). In this climate regime, the temperature was constant at 24 °C during the light period and at 20 °C during the dark period, respectively. RH was 60% during the light and the dark period and a light/dark photoperiod of 13:11 h was used. In this experiment, plants were transferred to “chamber 1” one day before growth measurements started. The leaf area growth of terminal leaflets of the second to the fourth trifoliate leaf was measured simultaneously on six plants for around one week.

In both experiments, young and most recently unfolded terminal leaflets of six plants were fixed separately in a metal frame (Figure 4.8B) by gluing strings (Dyneema® fibers, 0.16 mm in diameter, Climax, Ockert GmbH, Puchheim, Germany) to the margin of the leaflets and tautening the strings over a second metal ring with weights of each around 10 g. White plastic beads (8 mm in diameter) were threaded onto the strings close to the margin of the leaflets to serve as artificial landmarks for the later tracking. On top of each
leaflet, a monochrome CMOS camera (DMK 23GP031, maximal resolution of 2592 × 1944 pixels, The Imaging Source Europe GmbH, 28215 Bremen, Germany) with a narrow bandpass interference infrared filter (940 nm) was installed. To allow image acquisition during the dark period, a ring with six infrared light-emitting diode (LED) clusters (940 nm) was installed and a black background was placed under the leaf for an optimal contrast of the images for the later tracking of the white beads (Figure 4.8A-C). Images of each leaflet were taken every 90 s for around one week until the measurements of the next leaflets started. During all measurements, temperature and RH was monitored with two data loggers (HOBO® UX100-003 Temperature Relative Humidity Data Logger, Onset Computer Corporation, USA). The photoperiod was monitored with a light on/off logger (HOBO® UX90-002 Light On/Off Data Logger, Onset Computer Corporation, USA). The loggers were installed at around the height of the measured leaves.

4.5.1.3 Image processing

Image sequences were analyzed with the software ‘Martrack Leaf’ (see Mielewczik et al. (2013) for more detail). This software tracks the position of every bead in the complete image sequence. For every image the area of the polygon defined by the beads is calculated (Figure 4.8C). The relative growth rate (RGR) of the polygon area from one image to the subsequent image can be calculated and used as a proxy for the relative growth of the leaflet.

4.5.2 Leaf elongation growth of wheat

4.5.2.1 Experimental setup in the field

Measurements were conducted on the 5th to the 8th leaf of winter wheat [Triticum aestivum L., variety ‘CH-Claro’] in the field of the research station for plant science of ETH Zurich in Eschikon, Lindau (Switzerland) in 2013. A hair clip was fixed on the tip of a young leaf. On this hair clip, a string with two attached black beads was fixed that was going over a rod and taut by a counterweight. A white panel with a fixed ruler was placed in the background of the beads (Figure S4.5E). Nine leaves of separate wheat plants could be fixed in front of the white background. A weatherproof CCTV camera (INVID MK279IR, CMOS sensor, maximal resolution of 1600 × 1200 pixels, Pro-Store Technology GmbH,
Germany) with an internal infrared lighting was placed in a distance of around 2 m to the white panel and every 5 minutes an image of the panel was taken and saved on an internal storage (Figure S4.5D). Two of these measurement setups were placed in the field, enabling to measure the growth of 18 leaves simultaneously. The above described setup was similar to the method described in Nagelmüller et al. (2015; manuscript submitted in a revised version).

Image sequences were analyzed with a MATLAB® based (The Mathworks, Natick, MA, USA) algorithm described in more detail in Nagelmüller et al. (2015; manuscript submitted in a revised version). This software tracks the position of every bead in an image sequence with the same algorithm as used in ‘Martrack Leaf’ (Mielewczik et al. 2013). In a next step, the displacement (in pixels) of every bead is calculated from image to image. From the detected displacement in pixels and the known distances on the ruler, the leaf elongation rate (LER, mm h\(^{-1}\)) for the leaves is calculated.

### 4.5.2.2 Plant cultivation and experimental setup in the climate chamber

Winter wheat [Triticum aestivum L., variety ‘CH-Claro’] was sown in black polypropylene pots (11.3 x 11.3 x 21.5 cm, 2 l) filled with a commercial potting mix substrate (‘Spezialmischung 209’, RICOTER Erdauftbereitung AG, Aarberg, Switzerland) in a depth of around 2.5 cm. Plants were grown in a climate chamber of the same type as described in section 1.5.1.2 and watered three times per week. Two climate settings, differing only in the temperature were used for the measurement. In the colder setting, the temperature ranged between 2 and 12 °C and in the warmer setting between 15 and 25 °C (see table S4.6 and S4.7 in the supplemental material for more details). For both settings, relative humidity was kept constant at 60% and light intensity ranged from 0 to 600 μmol PAR m\(^{-2}\) s\(^{-1}\).

Wheat leaf elongation in the climate chamber was measured by clipping a hair clip on the tip of the third leaf at the time when it reached a length of around 2 cm (Figure S4.5C). The clip in turn was attached to a nylon string that was placed over a pulley of a rotary resistance transducer (RRT) and taut by a counterweight (Poiré et al. 2010b). The pulley is rotated by the leaf growth and the RRT measures the change in voltage in mV (measured every second and saved as 5 minute mean) that is recorded on a data logger (CR200,
Campbell Scientific Ltd., UK). The recorded values in mV were linearly converted (conversion factor of 0.018 mm mv\(^{-1}\) h\(^{-1}\)) to mm h\(^{-1}\) after manual calibration of the device. LER (mm h\(^{-1}\)) of ten leaves were measured simultaneously (Figure S4.5A+B). All plants were grown under the warm climate conditions (Table S4.7) until the second leaf appeared. These climate conditions were also kept during leaf growth measurements for the warmer setting. However, for the colder setting, the temperature was lowered during the dark period to 2 °C during a period of twelve hours as soon as the second leaf appeared and from then on the colder setting (Table S4.6) was programmed. During leaf growth measurements, temperature and RH was monitored with a data logger (HOBO® U10-003 Temperature Relative Humidity Data Logger, Onset Computer Corporation, USA).

4.5.3 Statistics

All statistical analyses were conducted in R (R Core Team 2015). In experiment I, for each day and every measured leaf the mean growth rate during the light period and during the dark period and the respective leaf area relative to fully grown leaves were calculated. To check for a correlation of these data, a Spearman correlation was calculated. After this, linear regressions were calculated for the light period with linear factors and for the dark period without linear factors, respectively (Figure 4.4). The difference between the relative growth rate (RGR; % h\(^{-1}\)) during the light period and the dark period was compared with a one-sided Welch’s t-test (Table 4.1). Additionally, the correlation between growth rate and temperature was examined. For this, the measurements were grouped by their leaf area relative to fully grown leaves and further divided in different time periods. In a next step, linear regressions for these groups were calculated.
Acknowledgments

The authors would like to thank Hansueli Zellweger for seeding, raising and taking care of the soybean and wheat fields at the ETH research station for plant sciences in Eschikon, Lindau and Norbert Kirchgessner for implementing different steps of the Leaf Length Tracker system. MF acknowledges support from the Swiss National Science Foundation (grant nr. 315230_144078/1).

List of author contributions

MF, LR, AB and AW designed the experiments. Experiments and analyses on soybean were performed by MF and LR. Experiments and analyses on wheat were performed by AB. Figures and tables were performed by MF and LR. MF drafted the manuscript with help and contributions by AW. All authors read and approved the final manuscript.
4.6 References


4.7 Supplementary material

Table S4.1. Climate settings during leaf growth measurements of *G. max* in experiment II. Temperature 1, humidity 1 and light intensity 1 were set for two days. Then, the settings were switched to temperature 2, humidity 2 and light intensity 2 and kept until the end of experiment II.

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Figure S4.2. Mean relative growth rate of *G. max* during the light period (white background) and dark period (grey background) in experiment I & II for TL 2 (A, B), TL 3 (C, D) and TL 4 (E, F). The black line shows the mean relative growth rate in experiment I. The orange line shows the mean relative growth rate in experiment II. The green line shows the point in time when the climate settings were switched from the simulated to the “conventional” conditions in experiment II.
Figure S4.3. Diel leaf movements of *G. max*. Images were taken with a closed-circuit television (CCTV) camera on a freely growing soybean plant during experiment I. The red line in the images indicate the leaf angle of one leaflet. Rising in the morning (A-C) and lowering in the evening (G-I) of the leaves. Constant leaf position during the light period (D-F) and during the dark period (J-L).
Table S4.4. Climate settings during leaf growth measurements of *G. max* in experiment I.

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Figure S4.5. Experimental setup to measure leaf length growth of *T. aestivum* in the climate chamber and in the field. (A) Overview of the setup in the climate chamber with ten rotary resistance transducers (RRTs). (B) Close-up view of the RRTs. (C) Close-up view of a hair clip attached to a leaf tip of *T. aestivum*. (D) Overview of the setup in the field with a weatherproof CCTV camera in front of the measurement panel. (E) Close-up view of the fixed leaves in front of the white background panel.
### Table S4.6. Cold climate settings during leaf growth measurements of *T. aestivum* in the climate chamber.

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### Table S4.7. Warm climate settings during leaf growth measurements of *T. aestivum* in the climate chamber.

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Chapter V

Terrestrial 3D laser scanning to track the increase in canopy height of both monocot and dicot crop species under field conditions

Michael Friedli, Norbert Kirchgessner, Christoph Grieder, Frank Liebisch, Michael Mannale and Achim Walter

ETH Zürich

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Abstract

**Background:** Plant growth is a good indicator of crop performance and can be measured by different methods and on different spatial and temporal scales. In this study, we measured the canopy height growth of maize (*Zea mays*), soybean (*Glycine max*) and wheat (*Triticum aestivum*) under field conditions by terrestrial laser scanning (TLS). We tested the hypotheses whether such measurements are capable to elucidate (1) differences in architecture that exist between genotypes; (2) genotypic differences between canopy height growth during the season and (3) short-term growth fluctuations (within 24 h), which could e.g. indicate responses to rapidly fluctuating environmental conditions. The canopies were scanned with a commercially available 3D laser scanner and canopy height growth over time was analyzed with a novel and simple approach using spherical targets with fixed positions during the whole season. This way, a high precision of the measurement was obtained allowing for comparison of canopy parameters (e.g. canopy height growth) at subsequent time points.

**Results:** Three filtering approaches for canopy height calculation from TLS were evaluated and the most suitable approach was used for the subsequent analyses. For wheat, high coefficients of determination (R²) of the linear regression between manually measured and TLS-derived canopy height were achieved. The temporal resolution that can be achieved with our approach depends on the scanned crop. For maize, a temporal resolution of several hours can be achieved, whereas soybean is ideally scanned only once per day, after leaves have reached their most horizontal orientation. Additionally, we could show for maize that plant architectural traits are potentially detectable with our method.

**Conclusions:** The TLS approach presented here allows for measuring canopy height growth of different crops under field conditions with a high temporal resolution, depending on crop species. This method will enable advances in automated phenotyping for breeding and precision agriculture applications. In future studies, the TLS method can be readily applied to detect the effects of plant stresses such as drought, limited nutrient availability or compacted soil on different genotypes or on spatial variance in fields.

**Keywords:** laser scanning, scan point cloud, canopy height growth, maize, soybean, wheat.
5.1 Background

Plant growth is a good indicator of crop performance and is measureable by different methods and on different spatial and temporal scales (Walter et al. 2009). Plant growth reveals detailed information about the state of a plant (Walter et al. 2015) and allows for the assessment of the tolerance of a plant to abiotic stress such as drought (Banziger et al. 1999, Lipiec et al. 2013), heat (Lipiec et al. 2013) or nutrient deficiency (Ha & Tran 2014). Today’s technologies offer many different possibilities to measure plant growth automatically, non-invasively and non-destructively. Many of these technologies construct a 3D scan point cloud of plants or canopies. A very simple approach to measure plant growth by taking images with a commercial digital camera was used for example by Sritarapipat et al. (2014) to observe plant height changes in a rice field. In more complex approaches 3D images of plants are reconstructed by using stereo cameras (Biskup et al. 2007), by analysing multiple images taken from different viewing angles (Paproki et al. 2012) or by taking depth images (Chéné et al. 2013). In recent studies, unmanned aerial vehicles (UAVs) were used to generate 3D reconstructions of winter wheat from multiple images to estimate crop height (Khanna et al. 2015) and to generate 3D digital surface models of barley from hyperspectral information (Aasen et al. 2015). In another study, a laser scanner was mounted on a UAV to estimate crop height of maize (Anthony et al. 2014).

A very interesting and precise technology is the so-called 3D digitizer which uses ultrasonic or electromagnetic devices (digitizing pens) to construct 3D images of plant parts or whole plants. Plant architecture of different crops was measured with 3D digitizers to calculate light models in plant canopies in rice (Zheng et al. 2008) and cucumber (Wiechers et al. 2011). 3D digitizing is very labour and time intensive because the digitizing pen needs to be manually pointed to important landmarks on the plant (for example leaf and shoot tips) to map plant architecture in 3D. Therefore, this technology cannot be used as an automated, high throughput phenotyping system.

A sophisticated technology, that is becoming more and more important, is the active remote-sensing laser rangefinder which uses a laser beam to determine the distance to
an object. Different principles for distance detection exist (for a review see Rosell & Sanz (2012) or Omasa et al. (2007)).

Terrestrial laser scanning (TLS) offers a unique opportunity to make non-invasive and non-destructive measurements of canopies to characterize plant growth and to analyze diverse architectural parameters. TLS measurements render point clouds that depict the surface of the visible canopy oriented towards the observing device. These point clouds can be further analyzed, which has been done already in the fields of (1) forest ecology; (2) precision agriculture; and (3) phenotyping.

So far, most TLS studies were conducted in forest ecology to measure tree height, volume, leaf area, biomass and other important plant parameters (Hopkinson et al. 2004, Parker et al. 2004, Clawges et al. 2007). Hosoi & Omasa (2009a) for example investigated the seasonal change of broad-leaved woody canopy leaf area density profiles. The plant structure and chlorophyll content in broadleaf saplings was studied by Eitel et al. (2010).

In precision agriculture, measurements of orchard volumes (Polo et al. 2009, Rosell et al. 2009) or leaf area in orchards (Polo et al. 2009) or viticulture (Arno et al. 2013) have been conducted. The geometric characterization of tree crops is important for a number of different aspects such as the application of pesticides or irrigation systems (see Rosell et al. (2012) for a review). The aspect of canopy characterization is also important in vineyards to improve pesticide application methods (Llorens et al. 2011). In field crops, TLS was applied to discriminate maize plants from weeds and soil for a targeted application of herbicides (Andujar et al. 2013). Sensing of the nitrogen status of wheat plants by TLS was used for improved application of nitrogen fertilizers (Eitel et al. 2011). In another approach, Saeys et al. (2009) used TLS to estimate crop density of wheat that could be used to automatically adjust the speed of a combine harvester for a constant intake of biomass.

Another important research field in which TLS is applied is plant phenotyping under lab or field conditions. Morphological plant parameters such as canopy height (Lumme et al. 2008, Tilly et al. 2014) and leaf area (Gebbers et al. 2011, Hosoi et al. 2011) have been investigated. Besides morphological parameters also structural (number of leaves, orientation of surfaces, topology) and functional information (photosynthesis, stomatal
Terrestrial 3D laser scanning to track the increase in canopy height has been studied (Sirault et al. 2013). Biomass (Ehlert et al. 2009, Eitel et al. 2014, Tilly et al. 2014) is probably the second most important parameter next to height growth (Lumme et al. 2008). In a newer approach, the detection of individual maize plants has been performed to improve plant growth models or crop management strategies (Hofle 2014). The relevance of TLS measurements for field research remained limited though, since TLS measurements were typically conducted on single plants in pots (Dornbusch et al. 2007, Paulus et al. 2014a) or on small plants like Arabidopsis thaliana (Kaminuma et al. 2004) from which conclusions to crops cannot easily be drawn. Furthermore, these measurements were often carried out under controlled and relatively artificial environmental conditions such as in climate chambers (Kaminuma et al. 2004) or greenhouses (Hosoi et al. 2011, Kjaer & Ottosen 2015). If TLS measurements were conducted in the field, this was done on very small areas (Hosoi & Omasa 2009b: 1 m²) or with a low resolution (Hoffmeister et al. 2013).

Elucidation of improved field management practices or of optimal genotypes in breeding programs needs to be done in plots and plant canopies of a relevant size in the field. Therefore, it was the overall aim of this study to analyze the capability and the limits of TLS approaches (Figure 5.1) in the field on plot areas of several dozen to hundreds of m² in different crops (Figure 5.2; wheat, maize and soybean) that are of relevance to global agriculture. Precise knowledge of these capabilities and limits is necessary to better connect the multitude of small-scale experiments under controlled conditions with field studies and to come to conclusions of relevance for crop science with respect to the grand challenges of global climate change and sustainable intensification of agricultural practices.

Therefore, we tested the hypotheses, whether TLS field measurements are capable to elucidate (1) differences in architecture that exist between genotypes; (2) genotypic differences in canopy height growth during the season and (3) short-term growth fluctuations (within 24 h), which could indicate e.g. responses to rapidly fluctuating environmental conditions.
Figure 5.1. Experimental set-up in the soybean field. (A) The set-up in a soybean field with the laser scanner on an elevator tripod and white spherical targets to merge the single scans into a 3D point cloud; (B) Close-up view of „Faro Focus 3D S 120” laser scanner; (C) Close-up view of white spherical targets on aluminium rods.
Figure 5.2. Plant height maps (bird’s eye view) computed from TLS. Black rectangles indicate ROIs and red circles the spherical targets. (A) Wheat field; (B) Maize field; (C) Soybean field.
5.2 Results

5.2.1 Correlation between manually and TLS-derived wheat canopy height

Conceivably, outliers at the top of the raw data point cloud can lead to erroneous interpretations of canopy height (Figure 5.3). Therefore, three filtering approaches were conducted with the aim to identify optimal filtering approaches for subsequent tasks (see Material and Methods for more details). In order to perform this quality check, the coefficients of determination ($R^2$) of the linear correlations between the manually measured reference height and three filtering approaches (FAs) for the TLS-derived canopy height were evaluated for the 100th (P100) to the 90th percentile (P90) of the investigated regions of interest (ROIs) for three measurement dates (Figure 5.4A-C). For the three FAs, $R^2$ reached highest values for the last measurement date. At this date, the canopies of the wheat plots were denser and reached canopy closure. Therefore, the laser beam could not penetrate very deep into the canopies. Thus, most of the scan points were located on top of the canopy.

Figure 5.3. Calculation principle of height maps and statistics. In a first step, 3D points (points_3D) are projected to xy-plane (points_proj). Then, for each ROI (pixels for height maps) the contained points_proj are determined. Then, the z-coordinates of points in 3D which correspond to the ROI are known and can finally be used for further processing or statistical evaluation.
Terrestrial 3D laser scanning to track the increase in canopy height

Figure 5.4. Correlation between manually measured and TLS-derived canopy height of *T. aestivum* for three dates. Coefficients of correlation for manually measured and TLS-derived canopy height of *T. aestivum* shown for the 100th to 90th percentile for three filtering approaches (FA): (A) FA_{ALLPOINTS}; (B) FA_{MEDIANMAX}; (C) FA_{MEDIANP99}. Regression between manually measured (reference height) and TLS-derived canopy height (calculated from the 99th percentile for each FA) of *T. aestivum*: (D) FA_{ALLPOINTS}; (E) FA_{MEDIANMAX}; (F) FA_{MEDIANP99}. n=192 per date.
The investigated ROIs were analyzed by testing three filtering approaches with different percentiles. The first FA used all contained scan points of each ROI and calculated certain percentiles of the z coordinate. The second FA filtered the ROI patchwise by a weighted median of certain percentiles. Thereby, the full ROI was considered, even if extreme values were present as for example in very heterogeneous field situations with a few very high plants. See section 5.5.3 for a detailed description of the calculation of FAs. In the FA, which included all points of a ROI in the calculation (FA_{ALLPOINTS}), R^2 for P100 was lowest for all measurement dates (Figure 5.4A). This percentile included all maximum points and as a consequence also potential outliers that contributed to the low R^2. The values of R^2 for the first two measurement dates gradually decreased from P99 to P90. At these two dates, the canopy was not yet closed, so that the laser beam reached lower parts of the canopy. Thus, the values of the lower percentiles did not depict the maximum but lower parts of the canopy.

In the FA using the weighted median of the maxima of each patch in a ROI (FA_{MEDIANMAX}), R^2 values of P100 for the three dates were also slightly lower than the ones of P99. R^2 values for the last measurement date did not markedly vary between P99 and P90. The values of R^2 for the first two measurement dates decreased from P99 to P90 and were always lower for the first date.

In the FA using the weighted median of P99 of each patch in a ROI (FA_{MEDIANP99}) the highest values for R^2 were obtained for the last measurement. However, the values were clearly lower for this FA compared to the other two FAs. The values for R^2 of the first two dates were very similar from P100 to P96 but then diverged with decreasing percentiles below P95. The values of R^2 for the first measurement were more or less uniform throughout the entire tested range of R^2, whereas the values of R^2 for the second measurement date decreased towards lower percentiles.

For the three evaluated FAs, P99 resulted in the highest values for R^2 throughout the season. Therefore, P99 was considered as the TLS measure which approximated canopy height most realistically. In the next step, FAs were compared with each other on the basis of P99 values of TLS-derived canopy height that were plotted against manually measured reference heights (Figure 5.4D-F). For FA_{ALLPOINTS}, R^2 increased from 0.86 for the first date.
Terrestrial 3D laser scanning to track the increase in canopy height

to 0.92 for the last date (Figure 5.4D). For FA\textsubscript{MEDIANMAX}, R\textsuperscript{2} increased from 0.85 for the first date to 0.93 for the last date (Figure 5.4E). For FA\textsubscript{MEDIANPP99}, R\textsuperscript{2} was comparable for the first (R\textsuperscript{2} = 0.73) and the second date (R\textsuperscript{2} = 0.73) but was higher (R\textsuperscript{2} = 0.76) for the third date (Figure 5.4F). Using the three dates combined in a regression, FA\textsubscript{ALLPOINTS} (R\textsuperscript{2} = 0.99) and FA\textsubscript{MEDIANPP99} (R\textsuperscript{2} = 0.98) reflect higher coefficients of determination than FA\textsubscript{MEDIANMAX} (R\textsuperscript{2} = 0.95). Therefore, FA\textsubscript{ALLPOINTS} was used in subsequent calculations.

Using FA\textsubscript{ALLPOINTS}, the correlation between manually and TLS-derived canopy height growth was then analyzed for wheat as well. Growth can be calculated by the difference of the canopy height for a certain plot at two subsequent dates. Differences of manual height measurements and differences of TLS-derived canopy height were then put in relation to each other for different measurement periods. For the period from the first to the second measurement, the increase in canopy height was not large, which resulted in a relatively low R\textsuperscript{2} of 0.21 (Figure 5.5). For the period from the second to the third measurement, the increase in canopy height for all genotypes was - according to the manual reference measurements - between 0.2 and 0.6 m. For this period, a high R\textsuperscript{2} of 0.80 was obtained for FA\textsubscript{ALLPOINTS} (Figure 5.5). It has to be noted that the values for the first period seem to be approximated by the correlation obtained for the second period in a reliable manner.

![Graph](image)

Figure 5.5. Correlation between canopy height growth of *T. aestivum* from manually measured (reference) and TLS-derived canopy height. Canopy height growth was calculated for the periods from 09.04.2014-15.04.2014 and 15.04.2014-19.05.2014 using FA\textsubscript{ALLPOINTS}. n=192 per date.
5.2.2 Short-term canopy growth

Maize displayed a diel (24 h) growth pattern that followed temperature (Figure 5.6). This growth pattern obtained from TLS was confirmed by manual height reference measurements and was observed during both measurement campaigns in June and July. Highest growth rates were found in the afternoon when the temperature reached its peak and lowest growth rates were observed during the night when temperature was lowest. In the morning, intermediate values for growth rate and temperature were obtained, respectively. No obvious difference in growth was observed between the two genotypes. In July, the growth rate for both genotypes was nearly twice as high as in June. The different growing stages at the two measurement campaigns and also the faster increase in temperature during the morning in July can probably explain the different growth rates. For most of the measurement points, the reference measurements were higher compared to the TLS-derived values.

![Figure 5.6. Maize canopy height growth. Canopy height growth (mm h⁻¹) of maize computed from TLS (99th percentile of FA_ALLPOINTS) and measured manually (reference) for the two varieties ‘Gottardo’ and ‘Bonfire’. Measurements were conducted in the year 2014: (A) 21. & 22. June; (B) 16. & 17. July. Air temperature is shown as a green line. Shaded areas indicate the period between sunset and sunrise.](image-url)
For soybean, the obtained canopy height from TLS increased from the morning to the afternoon but then decreased again towards the evening (Figure 5.7A+B). This observed pattern of the canopy height corresponded to the diel (24 h) movement of soybean leaves (Figure 5.8) and can be seen very well for the measurement campaign conducted in July. The increase in height from the afternoon of one day to the afternoon of the next day clearly indicated canopy height growth. Manual reference measurements showed a continuous increase in canopy height over the measured period of 36 hours (Figure 5.7A+B). Differences between canopy height values obtained by TLS and manual reference measurements can mainly be explained by different approaches used to obtain these values: P99 of TLS mainly quantifies the height of the uppermost leaf tips; manual measurements quantify shoot height, which is lower. Manual measurements indicated a clear diel fluctuation of height growth with a similar pattern as in maize, largely following the temperature (Figure 5.7C+D).

**Figure 5.7. Soybean canopy height and height growth.** (A, B) canopy height (m) of soybean computed from TLS (99th percentile of FAALLPOINTS) and measured manually (Reference) for the two varieties ‘Gallec’ and ‘Lissabon’. (C, D) canopy height growth (mm h⁻¹) of manually measured soybean plants (n=10 per genotype and time point) of the varieties ‘Gallec’ and ‘Lissabon’. Red and blue lines indicated the canopy growth rate from afternoon to afternoon calculated from the computed TLS data. Measurements were conducted in the year 2014: (A, C) 21. & 22. June; (B, D) 16. & 17. July. Shaded areas indicate the period between sunset and sunrise.
Figure 5.8. Soybean leaf movement during a day. Images to illustrate movement of soybean leaves and changing canopy height on 25.07.2014: (A) 8 am; (B) 3 pm; (C) 8 pm. The two plots on the left and right to the closest aluminium rod were sown with the varieties ‘Gallec’ and ‘Lissabon’, respectively.
5.2.3 **Plant architectural traits**

TLS of the “height level experiment” in maize showed that scanning of the whole, intact canopy revealed positions of the different plant organs in a relatively reliable manner (Figure 5.9, violet line). Shoulders (local maxima) in the scan point height distribution (SHD) of the intact canopy corresponded well to leaf and ear positions in the subsequent scans of the “height level experiment” (dashed lines in Figure 5.9). Of course, leaves positioned lower in the canopy became more pronounced, when leaves on the top were cut and dismissed. This was true for both genotypes which reflected only small differences between each other.

5.2.4 **Deviations of transformed positions of spherical targets**

Small deviations of transformed positions of spherical targets in wheat (0.0084 ± 0.0039 m), maize (0.0042 ± 0.0031 m) and soybean (0.0021 ± 0.0006 m) were achieved (Table 5.1). These deviations include technical measurement limitations of the laser scanner and potential movement of the positions of spherical targets throughout the field season.

<table>
<thead>
<tr>
<th>Species</th>
<th>Average deviations of transformed positions (in m ± standard deviation)</th>
<th>Number of transformed sphere positions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Triticum aestivum</em></td>
<td>0.0084 ± 0.0039</td>
<td>24</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>0.0042 ± 0.0031</td>
<td>60</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>0.0021 ± 0.0006</td>
<td>60</td>
</tr>
</tbody>
</table>
Figure 5.9. Scan point height distributions (SHDs) and height levels of reference measurements. Colored lines show the SHDs for the different height levels derived by step wise cutting of the canopy (violet: H1, blue: H2, dark green: H3, bright green: H4, yellow: H5, orange: H6, red: H7). Dashed lines with numbers stand for the average reference measurements of different plant parts. (1: height of the whole plant, 2: flag-leaf, 3: second leaf, 4: third leaf, 5: forth leaf, 6: ear-leaf, 7: ear). Data are shown for the maize genotypes ´Bonfire´ (A) and ´Poya´ (B). Overview of the different height levels derived by stepwise cutting of the canopy (C) (maize drawings adapted from www.openclipart.org); for details see table 5.3.
5.3 Discussion

So far, most TLS studies conducted very detailed (Paulus et al. 2013) and mostly indoor (Kaminuma et al. 2004, Paulus et al. 2014b) measurements or they were carried out on large areas in the field (Hammerle & Hofle 2014) with often low spatial resolution. Time intervals between measurements were often in the range of weeks if ever several scans were made and until now, to our knowledge, no one examined the temporal resolution limits of TLS on canopy height growth of crops in the field. With our approach, we fill the gap between these two extremes. We obtained a better resolution as for example in (Hammerle et al. 2014) and therefore increase the applicability of TLS for breeding-related phenotyping and precision agriculture. For breeding, many different genotypes planted often on relatively small plots of a few square meters need to be characterized with respect to their performance and their reaction towards alterations of environmental parameters (White et al. 2012, Walter et al. 2015). Further, our approach can be used as ground truth calibration method for new measurement systems from e.g. UAVs developed for precision agriculture (Khanna et al. 2015, Aasen et al. 2015). Aasen et al. (2015) obtained an $R^2$ of only 0.7 and a constant underestimation of 0.19 m of the plant height from the UAV-based data compared to manually measured plant heights. In Anthony et al. (2014) the height measurements from UAV even indoors had a measurement error of above 3.5 cm. UAVs produce wind (downwash) by themselves that can move plant canopies when flying at low altitudes. Therefore, a certain distance of UAVs from the plant canopy is needed to exclude any influence on the plant canopy. Increasing the flight altitude, however, decreases the resolution of measurements and thus also the accuracy of plant canopy reconstructions. Therefore, our high precision method ($R^2$ of 0.93 for FA\textsc{ALLPOINTS} for the last date and $R^2$ of 0.99 for FA\textsc{ALLPOINTS using the three dates combined}) could be used to calibrate such systems. The fixed position of the spherical targets during the whole season on aluminium rods and solid ground screws is new in plant science under field conditions and at the same time a simple approach, which leads to a high precision of the measurement and therefore allows for comparison of canopy parameters (e.g. canopy height growth) at subsequent time points. In our approach, the spherical targets define a coordinate system that is fixed during the complete season and that can be used to transform scan point clouds from different
measurement dates into one and the same reference coordinate system. The small deviations of transformed positions of spherical targets are a strong evidence for the high accuracy of our measurement setup (Table 5.1). These deviations include technical measurement limitations of the laser scanner and potential movement of the positions of spherical targets throughout the season and are at the same time giving a value for the best achievable accuracy of our TLS approach. In our approach only the spherical coordinates have to be known and no additional expensive device such as a tachymeter or a GPS, as e.g. used by Aasen et al. (2015), is needed to measure the exact position of targets at each measurement date. Such measurement devices have their inherent technical resolution limits and sources of error that may result in an accumulation of inaccuracies during the season.

By evaluating different FAs for calculating canopy height from TLS data, we could show that in the FA\textsubscript{ALLPOINTS}, the 99\textsuperscript{th} percentile is best suited for computing the wheat canopy height (Figure 5.4). This FA has, compared to other studies (Hammerle et al. 2014, Tilly et al. 2014), the advantage that outliers are excluded from calculations and thus the risk of over- or underestimation of the canopy height can be reduced. Another benefit of this FA, compared with FA\textsubscript{MEDIANP99} is that no potentially important points are a priori excluded from the calculation. The temporal dynamic of the canopy is also clearly visible in the progress of the value of $R^2$: a lower canopy is more “susceptible” for underestimation of the canopy height depending on the chosen percentile.

In a recent study, Hammerle et al. (2014) investigated the effect of reduced point density of TLS crop surface models of wheat and rye. They examined the effect of a stepwise point reduction on the calculated canopy height from the maximum points or from the 90\textsuperscript{th} percentile (P90) compared with the original resolution and a low resolution scan. However, their low resolution scans and simulated reduced point clouds had only 30-50 points per m$^2$. For our purposes, this would have been by far a too low resolution to detect genotypic differences. Moreover, we could show for wheat (Figure 5.4) that – in younger growth stages – the real canopy height will be underestimated by using P90 and that using absolute maximum points for canopy height calculation involves the risk of including outliers.
The temporal resolution that can be achieved with our approach depends on the scanned crop. For maize that only shows slight leaf movements, a temporal resolution of several hours can be achieved by scanning e.g. three times per day. In contrast, soybean exhibits a strong diel leaf movement, resulting in a strong change of canopy height during a day (Figure 5.8). Thus, soybean is ideally scanned only once per day, after leaves have reached their most horizontal orientation. By taking the difference of the canopy height between two days at this time point, the real increase in canopy height will be detected. Otherwise, detected changes in canopy height are a jumble of daily leaf movements and real canopy height growth. Maize and soybean illustrate how important a sound knowledge about physiological processes of a scanned crop is. Growth of maize (Figure 5.6) and soybean (Figure 5.7C+D) follows temperature. For maize this is no surprise, as it is known from literature, that growth of monocot species follows temperature (e.g. Poiré et al. 2010). For soybean, the observed growth pattern with the highest growth in the afternoon is in contrast to the notion that dicot species show their maximal growth activity in the beginning of the day (type 1) or at the end of the day (type 2) (Walter et al. 2009). For soybean it was shown in several studies (e.g. Ainsworth et al. 2005, Friedli & Walter 2015) that maximal leaf growth occurs towards the end of the night.

For maize, we could show that plant architectural traits are detectable by TLS with our method (Figure 5.9). The obtained scan point height distribution histograms indicate the height position of plant organs, such as leaves and ears and genotypic differences in light penetration properties as potentially affected by number of leaves, leaf area index or leaf angles. Neither for maize (Figure 5.6) nor for soybean (Figure 5.7) different growth patterns could be detected by TLS for different genotypes in this study. For the precise distinction of genotypes by TLS beyond canopy height detection further studies including more genotypic variance and more measurement points during the season will be needed.

With our TLS approach of data acquisition and data analysis, we established – compared to other TLS studies – a quite simple way of handling TLS data of field crops. Our TLS approach therefore can be considered as a valuable tool to measure the canopy height growth of different crops under field conditions. The high correlation between manually measured and TLS-derived canopy height of wheat is showing the high accuracy of our method (Figure 5.4). The fact, that we could measure the diurnal pattern of canopy height...
growth in maize is another strong evidence for the accuracy of our TLS method (Figure 5.6). However, there are some restrictions to perform meaningful measurements. No measurement can be conducted if it rains due to the laser scanner that is not completely weatherproof and also due to technical issues regarding the scattering of the laser beam on raindrops. However, many devices used for field phenotyping cannot be used during rain. During the scanning process, it should ideally not be windy to prevent a blurred point cloud of the scanned crop. The dependence on windless conditions, however, depends on the scanned crop and also on the developmental stage of the crop and the research question. The scanning of crops that are small, stiff and have less surface exposed to wind is less dependent on wind conditions. The scanning of younger and thus normally smaller plants is also less affected by wind. Wind speeds of 2 m s\(^{-1}\) are feasible as our approach uses the statistical percentile method and therefore has a certain robustness against deviations caused by wind.

### 5.4 Conclusion

The TLS approach presented here allows for measuring canopy height growth and architecture of different crops under field conditions with a high temporal resolution, depending on crop species. The approach will therefore be a valuable component of plant breeding programs. It can also facilitate the elucidation of stress-related plant responses in the field in a variety of plants. Furthermore, additional and new plant/crop parameters as for example canopy volume, leaf angle distribution (in the absence of wind) and height positions of key organs such as leaves and ears could be computed and analyzed by accordingly adjusting the scanning resolution and the distances between scanning positions.
5.5 Materials and methods

5.5.1 Laser scanner

Measurements were performed with a „Faro Focus 3D S 120“ laser scanner (Faro Technologies Inc., Laker Mary, USA) (Figure 5.1B). The scanner allows the acquisition of point clouds of 7.1 up to 710.7 million points (MP). The number of points corresponds to the resolution of the measurement. Different quality options that differ in the ranging noise and scan rate (Hz) at a certain resolution are available. Scans with higher quality acquire range data with increased observation time and less noise. The scanning range of the device is up to 120 m and the accuracy in 10 m distance is 2 mm. The device uses a laser beam at 905 nm and the “phase shift measurement technology” to detect distances. In this system, infrared laser light is sent out and reflected back to the system. The distance of an object to the scanner is measured by analysing the shift in the phase of the returning beam (Faro 2011). The scanner can measure 360° on the vertical axis by rotation of the head of the scanner and 300° on the horizontal axis by a rotating mirror. The scanner was mounted upside down on an elevator tripod (elevator tripod aluminium 3.8 m, 50 kg max. load, VARYTEC, Germany) at a height of about 3.5 m (Figure 5.1A). This resulted in typical distances between scanner and canopy of 2 to 10 m. The point distance of the used resolutions ranged from 0.6 to 1.2 mm at 2 m distance and from 3.1 to 6.1 mm at 10 m distance, respectively (see table 5.2 for more details). It is intended to use the scanner on an automated mobile platform (Kirchgessner et al. 2015).
Table 5.2. Overview of the scanned species, dates, TLS measurements per date, scan parameters and reference measurements.

<table>
<thead>
<tr>
<th>Species</th>
<th>Date</th>
<th>TLS measurements per date</th>
<th>Scan resolution / quality</th>
<th>Point distance at 2 m (in mm)</th>
<th>Point distance at 10 m (in mm)</th>
<th>Reference measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Zea mays</em></td>
<td>23.09.2013</td>
<td>7</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>27.03.2014</td>
<td>1</td>
<td>0.25 / 3x</td>
<td>1.227</td>
<td>6.136</td>
<td>canopy height</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>02.04.2014</td>
<td>1</td>
<td>0.25 / 3x</td>
<td>1.227</td>
<td>6.136</td>
<td>canopy height</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>09.04.2014</td>
<td>1</td>
<td>0.25 / 3x</td>
<td>1.227</td>
<td>6.136</td>
<td>canopy height</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>15.04.2014</td>
<td>1</td>
<td>0.25 / 3x</td>
<td>1.227</td>
<td>6.136</td>
<td>canopy height</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>09.05.2014</td>
<td>1</td>
<td>0.25 / 3x</td>
<td>1.227</td>
<td>6.136</td>
<td>canopy height</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>21.06.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>22.06.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>16.07.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>17.07.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
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<td>plant height</td>
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<tr>
<td><em>Zea mays</em></td>
<td>21.06.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>22.06.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
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<tr>
<td><em>Zea mays</em></td>
<td>16.07.2014</td>
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<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>17.07.2014</td>
<td>3</td>
<td>0.5 / 3x</td>
<td>0.614</td>
<td>3.068</td>
<td>plant height</td>
</tr>
</tbody>
</table>

5.5.2 Set-up in the field and data acquisition

Measurements were conducted in the field of the research station for plant science of ETH Zurich in Eschikon, Lindau in 2013 and 2014 (Table 5.2). Maize (*Zea mays*) and wheat (*Triticum aestivum*) as monocot species as well as soybean (*Glycine max*) as a dicot species were scanned periodically with the laser scanner throughout the season.

Fields were scanned from different positions at the same point in time in regular intervals ranging from several scans per day to weekly scans. White spherical targets (For maize and soybean: 14.5 cm in diameter, Laserscanning Europe GmbH, 39120 Magdeburg, Germany; For wheat: 30 cm in diameter; do-it-yourself product) were distributed in the scanned area to allow for the later merging of the single scans from the same point in time but from different positions of a field to a scan point cloud. These targets were mounted on aluminium rods (Figure 5.1C; 1.52 m in length for soybean and wheat; 3.02 m in length for maize; 3 cm in diameter for all crops) that in turn were fixed to ground screws (80 cm in length, Krinner GmbH, 3272 Walperswil, Switzerland). As spherical targets and aluminium rods are sensitive to environmental influences, they were only positioned in the field during measurement times. The ground screws were positioned...
within the rows to avoid any contact to machines. Thus, the position of the spherical targets remained constant during the season and defined a fixed coordinate system for all measurements. By transforming scan point clouds to this fixed coordinate system, scan point clouds throughout the season could be aligned for each crop, facilitating the comparison of the canopy at the different measurement points.

In 2013, the scanned maize field had a size of 24 m by 17 m and consisted of 8 plots with a length of 8 m and a width between 5.25 to 6.75 m corresponding to 8 and 10 rows, respectively. The two varieties ‘Bonfire’ and ‘Poya’ (DSP, Delley Switzerland) were sown each on four of these plots. Eight white spherical targets were distributed over the maize field. A “height level experiment” was performed to test the hypothesis that the scanning of the whole canopy allows for the detection of leaf and ear height levels from maize plants. This was done by scanning a subplot containing four rows of ‘Bonfire’ and ‘Poya’, respectively from four positions on the 23.09.2013. After this, the plants were cut down step wise (removing first the tassel, then the flag leaf, then the second leaf from the top), each cut followed by the next scans (Table 5.3). With this procedure seven height levels were scanned in total (Figure 5.9). Manual height reference measurements were taken on ten maize plants for each variety and height level, respectively.

<table>
<thead>
<tr>
<th>Height level</th>
<th>Cut parts</th>
<th>Cutting point</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>Non</td>
<td>Non</td>
</tr>
<tr>
<td>H2</td>
<td>Tassel and flag leaf</td>
<td>Above second leaf</td>
</tr>
<tr>
<td>H3</td>
<td>Second leaf</td>
<td>Above third leaf</td>
</tr>
<tr>
<td>H4</td>
<td>Third leaf</td>
<td>Above forth leaf</td>
</tr>
<tr>
<td>H5</td>
<td>1-3 leaves</td>
<td>Above the ear</td>
</tr>
<tr>
<td>H6</td>
<td>Ear leaf</td>
<td>Above the ear</td>
</tr>
<tr>
<td>H7</td>
<td>Ear</td>
<td>Below the ear</td>
</tr>
</tbody>
</table>

In 2014, the scanned part of the maize field had a size of 6 m by 6 m and consisted of eight rows with a row spacing of 0.75 m. The two varieties ‘Bonfire’ and ‘Gottardo’ (KWS Saat SE, Einbeck, Germany) were sown each in four rows (Figure 5.2B). Five white spherical targets were distributed over the scanned area and at each date, scans from the four corners were carried out at around 6 am, 1 pm and 7 pm (6 pm in July). The scanned part of the soybean field had a size of 6 m by 6 m and consisted of four plots with a size of 1.5 m by 6 m. The two varieties ‘Gallec’ (DSP, Delley Switzerland) and ‘Lissabon’ (fenaco
Genossenschaft, Bern, Switzerland) were sown each on two plots (Figure 5.2C). Five white spherical targets were distributed over the scanned area and at each date, scans from the four corners were conducted at around 8 am, 3 pm and 9 pm (8 pm in July). The wheat field had a size of around 30 m by 40 m (Figure 5.2A). Seven white spherical targets were distributed over the whole wheat field and the field was scanned from 16 positions distributed homogeneously over the field. For later analysis only a part of the field (around 24 m by 24 m), including 192 plots with each a size of 1.5 m by 1.7 m, was used. 156 different genotypes were sown in these 192 plots (see Grieder et al. (2015) for more details).

Manual height reference measurements in soybean and maize in 2014 were taken during the first and the last scan on ten plants per genotype. In maize, the distance from a nail head in the soil next to the plant and the tip of the youngest leaf, which was manually straightened into an upright position, was measured. In soybean the distance from a nail head in the soil next to the plant and the tip of the shoot axis was measured. Manual height reference measurements per plot in wheat in 2014 were taken by holding a yardstick in the canopy at three positions and reading the value.

5.5.3 Data processing and data analysis

At the beginning of the season a measurement of soil level is done, afterwards measurements for plant heights can be performed. After the automatic detection of the spherical targets, single scans from each measuring date were registered according to the targets and with the use of the inclinometer in the software “FARO SCENE” (Faro Technologies Inc., Laker Mary, USA). Computed scan point clouds were exported as xyz-files (ascii format) and later processed with custom MATLAB® (The Mathworks, Natick, MA, USA) functions. Evaluation was done with an off-the-shelf computer (Intel® Core™ i7-3770 processor, 24 GB installed memory). The software together with a manual and example data can be downloaded from SourceForge (http://sourceforge.net/projects/cahst4tls). To reduce the file size and speed up the subsequent data analysis xyz-files were converted to mat-files (MATLAB®, binary data format). In the following the points contained in the 3D point clouds are always called scan points. These are used to calculate height maps whose elements are named pixels.
For the generation of height maps percentiles of the z-coordinate were used as a statistically robust method (Anthony et al. 2014, Kjaer et al. 2015). The stepwise processing and analysis of the point clouds were done as follows:

1. **Point cloud transformation to the fixed coordinate system**: Sphere coordinates of all scans were manually exported to txt files. They were used to estimate the rigid coordinate transformation for all point clouds to fixed coordinates (Taati 2010). The deviations of transformed sphere coordinates of each sphere from different scans were saved to txt-files as they give a value for the best achievable accuracy. The scan point clouds were then transformed to the fixed coordinate system.

2. 
   a. (in case of soil level measurement) **Generation of soil height** as a “height image” (HS) with 5 mm pixel size (Figure 5.3). Therefore the height minimum was first determined on the pixel grid. Gaps were interpolated. The result was median-filtered with a patch size of 21 cm.
   b. (in case of plant height measurement) **Subtraction of soil level** from each point of the point cloud using the appropriate entry of HS. The appropriate pixels of HS were found by projection of the point cloud along the z-axis. All points which are projected on the same pixel belong to a column with quadratic base area of 5 mm x 5 mm. They were processed together in the further evaluation by calculating their percentiles (Figure 5.3).

3. **Selection of regions of interest** (ROIs) as individual areas or as a grid (Figure 5.2). Border rows were not within the selected ROIs to exclude border effects.

4. **Height analyses of the point cloud** were carried out with two classes of analysis approaches consisting of three filtering approaches (FAs) in total. The first class consisted of one FA (FA\textsubscript{ALLPOINTS}) and the second class of two FAs (FA\textsubscript{MEDIANMAX} and FA\textsubscript{MEDIANP99}), respectively:
   a. Percentiles of all points (FA\textsubscript{ALLPOINTS})
i. Calculation of the percentiles for each ROI by taking every point of the whole scan point cloud within the ROI into account.

b. Patchwise

i. Taking the maxima (or the x\textsuperscript{th} percentile (Px)) for each 5 mm x 5 mm pixel, a height map H\textsubscript{P} of the point cloud was calculated (Figure 5.3). Points higher than 10 cm were regarded as ”plant points”, lower points were neglected.

ii. Patchwise calculation of percentiles of H\textsubscript{P} (edge length 15 cm in direction of the row and full row width for maize and 10 cm x 10 cm for soybean and wheat). Number of plant pixels N per patch was saved for later “weighting”.

iii. Calculation of the weighted median of percentiles per ROI. To calculate the weighted median of a percentile of a ROI each Px of all patches of the ROI was put N times to a list of which the median was calculated. Therefore patches with high plant coverage were stronger weighted than those with low plant coverage. In this study we applied the maximum value and P99 filter (FMEDIANMAX and FMEDIANP99)

5. **Calculation of canopy height and growth** or other parameters
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MF, CG, FL, MM and AW designed the experiments. Experiments were performed by MF, CG and MM. NK designed the measurement concept and implemented the analysis process in MATLAB®. MF drafted the manuscript with help and contributions by NK, FL and AW. Analyses, figures and tables were mainly performed by MF and partially by MM and NK. All authors read and approved the final manuscript.

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5.6 References


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Chapter VI

General discussion
Growth is a good indicator for the performance of a plant in a given environment. In the future, environmental conditions will change due to climate change (Solomon et al. 2007). Moreover, human population will increase to 9.7 billion people in 2050 (UN 2015). Therefore, agriculture has to be adapted to cope with predicted climate conditions and agricultural production has to be increased in the future. To achieve this, plant genotypes have to be selected that can cope with this changed environmental conditions and additionally produce higher yields. Plant phenotyping is needed to select such genotypes. I propose that in breeding programs this is done by monitoring the change of plant phenotypic traits such as growth rates. Nowadays, a lot of work and money is invested in plant phenotyping systems. A big proportion of measurements however is conducted under controlled conditions that seldom simulate the situation as it can be found outside in the field. Thus, this thesis focused on the establishment, improvement, application and evaluation of methods to measure plant growth on different organizational levels from the single leaf to the plant stand in the field.

6.1 Leaf growth measurements

So far, available image-based methods to measure leaf growth at a high temporal resolution were only usable under controlled conditions due to their high sensitivity to fluctuating illumination conditions (e.g. Schmundt et al. 1998, Poiré et al. 2010a). As expressed in the previous chapters, plant phenotyping must be brought to the field for a correct assessment of the performance of plant genotypes in the field and the subsequent selection of genotypes best adapted for future environmental conditions or specific local climates.

With the established marker-based tracking approach (chapter II) and the adaptations made to the measurement setup, leaf growth measurements of soybean in the field and in the climate chamber under simulated field conditions were performed in chapter IV. In chapter III, leaf growth measurements of soybean under “conventional” climate chamber conditions were conducted. Results from the measurement in the climate chamber under simulated field conditions showed the same diel growth pattern as observed in the field with an increase of the growth at the beginning of the day and a maximum growth during the day and a relatively constant growth rate of around 0.5% h\(^{-1}\) during the night. This is
in contrast to the opinion that dicot species show their maximal growth activity in the beginning of the day (type 1) or at the end of the day (type 2), irrespective or even reverse to the temperature profile (Walter et al. 2009). Results from chapter IV are also in contrast to the growth pattern of *G. max* under controlled climate conditions cited in previous studies (Bunce 1977, Ainsworth et al. 2005) and in chapter III where highest growth occurred during the night. Further, results from chapter IV disagree with the knowledge of literature that dicot plant species have their inherent genetically controlled diel growth pattern, irrespective of the environment (Ainsworth et al. 2005). The known fact, that monocot species follow the temperature profile with their growth rate could be confirmed for our model monocot species wheat in chapter IV under controlled and also under field conditions.

In the studies cited in Walter et al. (2009) that were measuring growth of dicot species, none of them worked with temperatures lower than 19 °C during the dark period. Therefore, it is highly probable that the growth rate of dicot species coincides with the temperature curve to a certain threshold. If this limitation i.e. the low temperature disappears, a diel growth pattern irrespective of temperature is activated that can be explained by the circadian clock (McClung 2001, Farré 2012). Leaf growth, gene expression and plant metabolism are tightly controlled by the circadian clock. The results of chapter IV point out the enormous importance of nighttime (air) temperature not only for leaf growth, but most probably for underlying control processes of metabolism and gene regulation. Not only air temperature but also the temperature around the root zone can influence the diel leaf growth pattern. A strong effect of root temperature on the diel leaf growth pattern was reported for *Ricinus communis* (Poiré 2010a). In this study, leaf growth of *R. communis* mainly occurred during the night and was strongly inhibited during the day when the root-zone temperature was decreased below a threshold value. However, Ainsworth et al. (2005) reported that warming of the roots of soybean during 8 h by a temperature difference of 10 °C (25 °C instead of 15 °C) did not affect the diel growth pattern. Thus, the influence of the root temperature on the diel leaf growth pattern is probably species-dependent, but it could also be included in analyses of further experiments.
The influence of the attached weights is an often raised issue. Such an influence cannot be excluded completely. However, preliminary experiments in Mielewczik et al. (2013) showed that the weights did not affect the final leaf size or shape. Walter et al. (2002) could show for *Ricinus communis* that moderate tensile forces on a leaf with simultaneous prevention of leaf movements do not affect the intensity and temporal distribution of the overall growth rate compared to freely growing leaves. In other words, the diel leaf growth pattern was not changed by the attached weights.

Results achieved by leaf area growth measurements occasionally are criticized, since it gives information about the growth in two dimensions and no information about the growth of the plant volume that can be more meaningful with respect to increase of plant biomass. On canopy scale, however, the canopy cover is a direct result of leaf area growth. Estimation of canopy cover by digital imaging can be used to non-destructive assess early vigor-related traits (Mullan & Reynolds 2010, Grieder et al. 2015). Measurements in our group showed that the observed diel growth pattern for leaf area of soybean reported in chapter III coincides with the diel leaf volume growth measured by computed tomography (data not shown in this thesis; manuscript submitted). Thus, diel leaf growth patterns of soybean measured in this thesis are also valuable to make statements about the diel leaf volume growth patterns that perhaps better depict the increase in plant biomass. Further, precise diel leaf area growth patterns could be integrated in canopy growth models to refine such models, thereby allowing to more precise calculate for example the light distribution in plant canopies (Wiechers et al. 2011).

Although our current measurement setup only allows a rather low throughput, it showed to be a valuable tool to reveal diel leaf growth pattern adaptations of soybean plants to different night temperature regimes under controlled conditions. The method could also be used to measure leaf growth patterns of other dicot species. Currently, the preparation of our setup for leaf growth measurements is relatively time-consuming. Thus, improvements of the current measurement setup regarding time saving should be tackled in the future. Data evaluation is already quite automated and with a few additional algorithms it could be automated even more. In the field under the operation of the FIP (described in section 1.3) the measurement setup could be adapted in a way, that across the field several growing leaves are on fixed positions with attached beads and that the
camera mounted on the carrier system of the FIP – instead of one separate camera per individual leaf – is taking images of all fixed leaves in a given time interval in 24-hour operation. This time interval strongly depends on the research question and longer periods than the 90 seconds currently used would be likely sufficient.

### 6.2 Canopy growth measurements

For assessing the performance of plants not only growth of single leaves and single plants are important. The growth of the plant community as a plant stand in a field in the end is crucial for the resulting yield and thus agricultural production. By monitoring the development of the canopy, a good statement about the growth of the plant stand can be made.

Different methods from very basic approaches to highly sophisticated technologies exist to measure the height of crops in the field. One promising technology for high-throughput phenotyping is terrestrial laser scanning (TLS) that allows to automatically scan a field with a resulting so-called scan point cloud of the scanned surrounding in 3D. From this scan point cloud different characteristics of the plant stand and single plants can be obtained. The attainable characteristics depend on the spatial and temporal scanning resolution, the crop species, the developmental stage of the crop and the space between single plants.

In chapter V, TLS was applied to scan maize, soybean and wheat under field conditions. During the first two field seasons (2013 and 2014) the laser scanner was mounted on an elevator tripod that was moved manually to the different scanning positions. Measurements from these two field seasons provide the data basis for chapter V. During the growing season 2015, measurements of the soybean and maize field under the Field Phenotyping Platform (FIP) were conducted by TLS from the rope suspended carrier system. Under the FIP, the carrier system was automatically moved from one scanning position to the next one. Thereby, movement between the scanning positions was markedly faster and throughout the season the scanning was always performed from the same positions stored in the data system of the FIP. Preliminary data analysis of the field data from 2015 showed very promising results, especially for soybean (data not shown in
this thesis). However, data from 2013 and 2014 already showed the significance of TLS for making statements about the development of the canopy height and partially also about plant architectural traits.

So far, in most studies TLS was conducted very detailed (Paulus et al. 2013) and mostly indoor (Kaminuma et al. 2004, Paulus et al. 2014) or measurements were performed in the field on large areas with often low spatial resolution (Hammerle et al. 2014). In most studies, the time intervals between measurements were in the range of weeks, if ever several measurements were made. With our approach, we filled the gap between these extremes. We performed TLS in the field with a high enough spatial and temporal resolution to study the temporal resolution limits of TLS on canopy growth of different crops in the field. Our setup in the field with the fixed ground screws, where aluminium rods with spherical targets were mounted is a new and at the same time simple approach. With this fixed position of the targets during the season, a high precision is achieved and these targets define a fixed coordinate system that facilitates the later canopy growth analysis. Thus, no expensive devices were needed for precise measurements of the positions of targets.

Different filtering approaches were tested to calculate the canopy height from TLS data, from which one proved to be suitable. This approach has, compared to the calculations of canopy height in other studies, the advantage that outliers are excluded from calculations but on the other hand potential important points are not excluded a priori from calculations. With these properties, the risk of over- or underestimation of the canopy height can be reduced.

The temporal resolution limit of our TLS approach depends on the scanned crop. For maize, a temporal resolution limit of several hours can be achieved, so that maize could be scanned e.g. three times per day. This high temporal resolution is mainly achieved due to low leaf movements shown by this monocot species. Soybean, in contrast to maize, shows strong diel leaf movements that result in strong changes of canopy height during the day. Thus, soybean is ideally only scanned once per day, after leaves have reached their most horizontal orientation. These results show, that it is important to know physiological peculiarities of scanned crops. Interestingly, canopy growth of maize and
soybean coincided with temperature profiles. For maize as a monocot species this is not surprising (e.g. Poiré et al. 2010b). For soybean, however this finding with the highest growth in the afternoon is in contrast to opinion about the growth pattern of dicot species (Walter et al. 2009) but it reinforces the results achieved in chapter IV for the diel leaf growth pattern of soybean. For maize, it was shown additionally that plant architectural traits are detectable by our TLS approach. In the obtained scan point height distribution histograms, the height positions of plant organs such as leaves or ears are indicated.

With our TLS approach of data acquisition and data analysis, we established – compared to other TLS studies – a quite simple way of handling TLS data of field crops. Our TLS approach showed to be a valuable tool to measure the canopy growth of different crops under field conditions. However, there are some restrictions to perform meaningful measurements. No measurement can be conducted if it rains due to the laser scanner that is not completely weatherproof and also due to technical issues regarding the scattering of the laser beam on raindrops. Depending on the chosen scan parameters, the time needed for data acquisition of a scanned crop can vary strongly. During the scanning process, it should ideally not be windy to prevent a blurred point cloud of the scanned crop. The dependence on windless conditions, however, depends on the scanned crop and also on the developmental stage of the crop. The scanning of crops that are small, stiff and have less surface exposed to wind are less dependent on windless conditions. The scanning of younger and thus normally smaller plants is also less affected by wind. In future experiments, the computation and analysis of plant parameters such as canopy volume, leaf angle distribution and height position of plant organs such as leaves and ears could be performed to make a better use of the captured scan point cloud.

6.3 Applications of phenotyping methods

During the last decade, enormous developments have been achieved particularly in image-based plant phenotyping. Thus, such plant phenotyping methods and their resulting description of plant x environment interactions are not only valuable to basic research but also to crop breeding and precision agriculture (Walter et al. 2015). Plant breeders nowadays have the opportunity to apply such plant phenotyping methods to select for desirable genotypes tolerant to different kind of abiotic stress such as the
drought tolerance in barley (Hartmann et al. 2011) or the salinity tolerance in Triticum (Rajendran et al. 2009).

In the future, it is conceivable that unmanned aerial vehicles (UAVs) (Zhang et al. 2013) are flying over crop fields several times throughout the season and collecting different information about the condition of the crops in these fields. This data could be used by farmers in the context of precision agriculture (Mulla 2013) for a sustainable application of fertilizers, pesticides or irrigation only on positions in the field where it is needed. This is just one aspect of agriculture of the future where digitization will be deployed to increase productivity and make work more efficiently. Animal nutrition in livestock farming is another field where digitization will play a more important role in the future.

6.4 Conclusions

Field-based plant phenotyping is needed because still today, experiments conducted under controlled climate conditions in climate chambers or greenhouses never simulate the situation as it can be found in the field. Results of our leaf growth measurements on soybean in chapter IV point out the enormous importance of nighttime (air) temperature not only for leaf growth, but most probably for underlying processes of metabolism and gene regulation. Thus, findings achieved under controlled conditions have to be interpreted with due diligence, especially if such findings are used to predict the performance of plants in the field.

Our methods were performed on our model plant species soybean, maize and wheat. Nevertheless, adaptations needed to use these methods for other crops should keep within limits. In the setups used in this thesis, our methods were applied on a rather low throughput. Therefore, in the future, the setups should be adjusted in a way that allows an application on a high throughput. In future experiments, the methods established in this thesis should ideally be applied with different genotypes under – from today’s agricultural perspective – non-optimal environmental abiotic and biotic conditions predicted for the future. In further experiments, the effect of simulated field conditions on processes of metabolism and gene regulation should be investigated. Furthermore, it would be interesting to study, if results achieved in short-term measurements (e.g. leaf
growth measurements over several days) are already meaningful enough to predict the performance of a genotype in the long-term i.e. over a growing season until the harvest with the respective yield.

The methods established and improved in this thesis could be used in the future by breeders to select superior genotypes but also by researchers to investigate different aspects of plant physiology. Furthermore, precise growth patterns measured in this thesis could be used to improve crop growth models. Thus, the results achieved in this thesis will hopefully contribute to improve the performance of genotypes of different crops to meet the needed increase in global food production in the future.
6.5 References


General discussion


List of abbreviations

3D: three dimensional
A. thaliana: Arabidopsis thaliana
CO₂: carbon dioxide
d: day(s)
DAS: days after sowing
FA: filtering approach
Fig.: figure
G. max: Glycine max
h: hour(s)
LA: leaf area
LAI: leaf area index
LED: light-emitting diode
LER: leaf elongation rate
n: size of statistical sample (statistics)
N. tabacum: Nicotiana tabacum
na: not available (data)
nm: nano meter
P: probability-value (statistics)
PAR: photosynthetically active radiation
R²: coefficient of determination (statistics)
R. communis: Ricinus communis
RGR: relative growth rate
RH: relative humidity
RLA: relative leaf area
ROI: region of interest
SE: standard error
T. aestivum: Triticum aestivum
Tab.: table
TL: trifoliate leaf
TLS: terrestrial laser scanning
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