Low temperature growth limits of alpine plants and winter crops

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1 General Introduction

Besides water shortage, low temperature is the second-most important climatic constraint to plant growth globally. This is reflected in the zonation of the world's biomes (Whittaker 1975). In seasonal climates of higher latitudes and elevations, low temperature comes into play in two ways: by constraining the length of the growing season and through cool conditions early and late within the season. These thermal constraints drive plant life in the wild (alpine, arctic, boreal) but they also affect winter crops such as winter wheat, barley or rye that are exposed to low temperatures for more than half of their seasonal life cycle. These winter crops present the most important carbohydrate source in the northern hemisphere (FAOSTAT 2015). Thus, understanding the thermal limits of plant growth in cold environments is a task for both plant ecologists and agronomists. In my thesis, I explore tissue growth in leaves and roots at very low temperatures in alpine taxa as well as in winter crops, both under field conditions.

1.1 Limiting low temperatures and thermal thresholds for plants in cold climates

It has been shown across many species and ecosystems that low temperature limits plant growth (Bliss 1956, Peacock 1975a, Chapin 1983, Baker and Younger 1986). The effects of low temperature can be expressed either by low temperature thresholds or by its gradual influence on the rate of growth. Thermal thresholds also define the beginning and end of the growing season as has already been known and quantified in the 19th century (De Candolle 1855, Harrington 1894, Smith 1920 in Gensler 1946). More recently, such thermal definitions have been applied to detect changes in the duration of the growing season (Carter 1998, Menzel and Fabian 1999, Frich et al. 2002, Robeson 2002). Others have used thermal thresholds to describe the productivity of grassland (Körner 2003, Wingler and Hennessy 2016) or to define temperatures for the growth and development of winter crops (Porter and Gawith 1999).

Most commonly, plant growth was found to become so slow at temperatures below 5 °C that it hardly contributes to biomass production. Plant growth under cool conditions can thus be predicted by the sum of temperatures above 5 °C (degree hours, °h; degree days °d, >5 °C). For example, the growing season has been defined as starting, the day after a daily mean temperature has exceeded 5 °C for five days in a row (Carter 1998, Frich et al. 2002). The position of the alpine treeline coincides with a growing season mean temperature of 6.4 °C globally (Paulsen and Körner 2014), which presumably also relates

to a common threshold for any significant rate of tree ring formation (xylogenesis) at around 5 °C in conifers (Rossi et al. 2007). However, if one zooms into that asymptotic approach toward zero growth, one may arrive at 2 °C as was shown for the alpine shrub *Rhododendron aganniphum* (Li et al. 2016). So defining such a zero point is also a matter of scale or resolution.

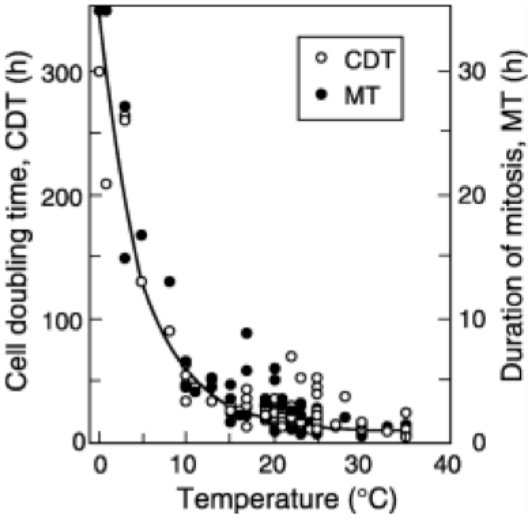
Both in agronomy and biogeography, thermal sums have been employed to define thermal limits. For instance, daily thermal sums above a 5 °C threshold define certain geographic vegetation limits in cold climates (Walker et al. 2005, Fang and Yoda 1989, Hou 1982). Similar low thermal thresholds were found when leaf of cold adapted plants were measured directly using the manual ruler method with a daily resolution. Leaf length increment was mainly observed at thermal sums above 5 °C in rapeseed (Körner 2008), sugar beet (Milford et al. 1985) or in perennial ryegrass (Peacock 1975). Moreover, the majority of root biomass of seedlings of temperate tree species and alpine herbs was produced above 5 °C soil temperature within the growing season (Alvarez-Uria and Körner 2007, Schenker et al. 2014; Nagelmüller et al. 2016a). Thus, temperatures between 5 and 7 °C were suggested as a likely thermal threshold for bulk root and shoot biomass production in cold climates (Körner 2003, 2012) or for crops (Körner 2008).

It is unclear what happens below these threshold temperatures when the production of new cells gradually reaches an absolute zero point. The task of this thesis was to identify such absolute thermal limits and the dynamics of growth below 5-7 °C at a high temporal resolution. A few studies have achieved measuring hourly growth rates in monocotyledons at such low temperatures, and zero points of growth for leaves have been extrapolated to occur between 0 and 1 °C in alpine grasses and winter cereals using the classical auxanometer approach (Körner and Woodward 1987, Gallagher and Biscoe 1979, Gallagher et al. 1979). Yet, the dynamics of growth close to these limiting low temperatures could not really be measured and are thus under-represented in the literature. However, such growth data are desirable to reveal growth constrains of plants at low temperature (Körner 2006). This doctoral thesis aimed at collecting continuous growth data in monocot and dicot plants in the narrow temperature range at which plant tissue production terminates.

1.2 Physiological and intrinsic limitations of plant growth at low temperature

Plant growth, is defined here as the net accumulation of dry matter and requires cell division, cell expansion and differentiation into different tissue types (Kost 2014) as well as sufficient carbon assimilation (source activity). Low temperature slows down plant metabolism. However, arctic-alpine plants and winter crops have adapted to lower optimal temperatures, and thus are able to grow in cold environments, and their growth at low temperature is commonly sink rather than source limited (Körner 2003, 2006, 2008). At 0 °C, cold adapted wild plants still achieve a rate of net-photosynthesis of ca. 30% of photosynthetic capacity (Tieszen 1975; Körner 2003). Similarly, winter wheat and rapeseed exhibit a relatively high net assimilation rate at low temperatures due to acclimated enzyme activity and are sufficiently provided with carbon for basic metabolism and growth (Hurry et al. 1995, Holaday et al 1992).

Figure 1.1



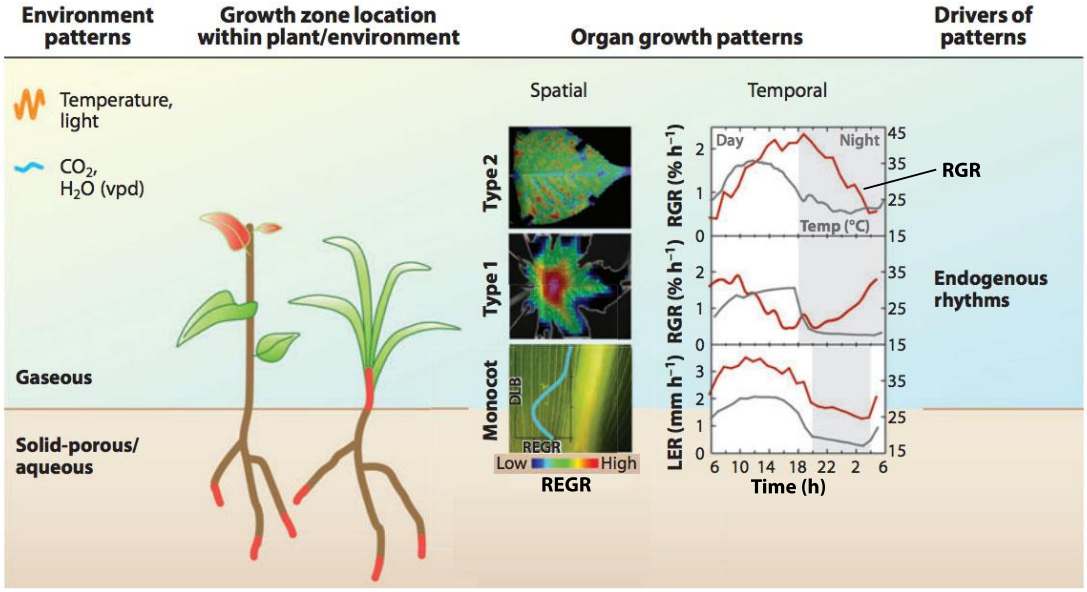
Literature survey of cell division, expressed by cell doubling time and duration of mitosis in relation to temperature. From Körner (2003)

Besides carbon assimilation, cell division is also not a growth-limiting factor at very low temperatures. The cell doubling time slows down towards 0 °C (Fig. 1.1) but cells in *Ranunculus glacialis* have been found in active dividing stage close to 0 °C using the metaphase arrest method (Francis and Barlow 1987; Körner and Pelaez Menendez-Riedl 1989; Körner 2003). Thus, the number of cells that enter the elongation and differentiation steps is believed not to be limited. Major drivers of cell expansion are turgor pressure, and water intake (formation of a central vacuole), both seemingly robust against the influence of low positive temperatures (Thomas et al. 1989; Pollock et al. 1990,

Fennell and Markhart 1998, Lee et al. 2005). Yet, cell expansion by water uptake requires simultaneous loosening of the cell wall and subsequent cell wall thickening to counterbalance turgor pressure as cells enlarge. By default, cell wall formation is suspected to be most sensitive to low temperature, and thus, should feed back on (or control) the cell cycle (Körner 2003, 2008). Moreover, lignification, required to build water-tight and functional xylem, is restricted below 4-5 °C in conifer tree ring formation (Rossi et al. 2007) and (at a finer resolution) suggested to find a limit even below 2 °C in alpine woody species (Li et al. 2016). Thus, cell wall formation and lignification are considered as very critical processes for growth limitation at low temperatures (Körner 2012). Plant tissues produced under very low temperatures should reflect these mechanisms by exhibiting shifts in the proportion of concurrently dividing and enlarging cells in root tips, and delayed lignification, a field for histological examinations (microscopy). Similar growth constrains are expected for leaves since both, roots and leaves are assumed to exhibit similar temperature responses given that all meristems are expected to employ the same processes at cell and tissue level.

Another important mechanism that regulates plant growth is the endogenous rhythm known as the circadian clock. These day-night cycles play an important role for plants to "sense" daily environmental fluctuations and to regulate their 24h-growth accordingly (McClung 2006 and Fig. 1.2). Such clock regulation can lead to increased photosynthesis by precise matching of the endogenous circadian clock and the day night periods (Dodd et al. 2005) or an optimized regulation of the starch metabolism (Graf et al. 2010). Circadian rhythms in interaction with environmental patterns also regulate the diel pattern of leaf growth with peak growth during the night as illustrated in Figure 1.2 (Webb 2003, Walter et al. 2009, Farré 2012, Ruts et al. 2012). Yet two types of dicot growth rhythms were found under controlled condition. Type 1 shows peak higher growth at the end and type 2 at the beginning of the night. It remains to be tested if such diurnal growth rhythms can also be found under low temperature field conditions. More evidence for clock related growth control are reported by transcriptome analysis showing that most genes involved in growth processes are expressed in a circadian rhythm (Covington 2008). However, circadian rhythms might be sensitive to low temperatures, which might disrupt circadian cycles, lower the amplitude of circadian clock components or change the expression of clock related genes, as was shown in *Arabidopsis thaliana* (Bieniawska et al. 2008). Thus, low temperature limitations of growth might also be caused by impaired endogenous rhythm, the exploration of which is also a task of the present thesis.

Figure 1.2



Overview of the relationship between temperature, growth zone positioning (red tissue), spatio-temporal growth patterns in leaves and roots, and drivers of growth patterns. Vapor pressure deficit, vpd; relative elemental growth rates, REGR; DLB, distance from leaf base. Modified figure taken from Walter et al. (2009)

Low temperatures and winter environmental conditions might also differently affect leaf growth of monocot and dicot plants due to fundamental morphological differences and positioning of the leaf growth zones (Fig. 2). In monocots the expanding tissue is restricted to the first millimeters above the meristem (Beemster et al. 1996, Ben Haj Salah and Tardieu 1996) and is sheltered against direct contact with the environment by the leaf sheath. In dicotyledons, the expanding leaf tissue is also assimilating and transpiring at the same time and is not sheltered by a leaf sheath and thus, much more exposed to fluctuating environmental conditions such as light, temperature and relative humidity. Most prominently, fluctuations of vapor pressure deficit with a peak at daytime can limit expansion of leaf tissue. This is due to excessive evapotranspiration and loss of turgor pressure even under well-watered conditions (Ben Haj Salah and Tardieu 1996, Tardieu et al. 1999). Situations with a high evaporative demand can occur frequently in alpine environments or in crop fields during winter even at low temperature induced by solar radiation.

1.3 The significance of field studies

All of my experimental work was conducted in the field. In the past, most experimental approaches at studying plant growth and related processes were made under climate chamber conditions because it is easier to precisely measure and analyze growth in relation to the environment when growth conditions are controlled. However, in the field, plants grow under highly variable environmental conditions, which makes it difficult to distil dose-response functions of growth and single environmental factors. Such field data is difficult to transfer to response functions obtained from climate chamber experiments to natural environmental conditions. Further, unrealistic temperature settings, limited light conditions and artificial growth substrates of finite volume constrain such scaling to 'real' life (Poorter et al., 2012a, 2012b, Porter et al. 2015). Moreover, in many climate chamber experiments, temperatures are kept constant or only change between day and night settings, and thus do not reflect natural environmental fluctuations.

Experimental data obtained under *in situ* field conditions allow the depiction of natural growth dynamics of plants but require deliberate and precise recordings of the temperature and other environmental conditions. Environmental conditions, particularly temperature, can vary enormously over small spatial distances, due to topography and exposure, with solar heating of both soils and the leaf layer causing massive departures from what adjacent weather stations might report (Scherrer and Körner 2011, Jones 2013). Therefore, temperature and other important environmental conditions such as relative humidity or light should be measured as close as possible to the growing plant organ. This was a key requirement during all growth measurement conducted in this thesis. In winter cereals (at least during the stocking stage) and in many alpine taxa, the position of the apical meristem is located in ca. 1 cm soil depth. In such cases, leaf growth might depend more in soil temperature than air temperature. Also the size of the sensor is critical because it determines the reaction time. To capture temperature fluctuations at high temporal resolution, sensors need to be small.

1.4 High resolution methodologies to measure plant growth

Any measurement technique applied to resolve the limits and dynamics of plant growth at low temperature and under field conditions need to fulfill a few basic requirements. Instruments have to be weatherproof and precise enough to record even small tissue increments close to the thermal limit, and measurements have to be performed continuously with high resolution to capture the effect of naturally fluctuating environmental conditions.

Plant growth is usually approximated by rates of extension or expansion of both roots and leaves. These are a proxy for growth, although there may be a certain delay between tissue volume gain and final dry matter content. However, any growth measured continuously as rates of tissue extension inevitably leads to an increase in dry matter over thermal time.

There are various approaches to measure rates of leaf extension growth, but their field applicability to achieve high-resolution data has clear limitations. Manual ruler measurements are easy and fast to perform in the field but do not reach the temporal and spatial resolution required to establish response functions. Measuring growth in terms of increasing volume has been achieved so far by computer tomography of root systems (Mooney et al. 2012) or whole plant 3D imaging techniques (Paulus et al. 2014). These techniques are not practicable for field use and measuring dry mass increase is impossible on a living plant. The development of techniques to achieve leaf growth measurements in the field date back to the early 20th century and are classical auxanometers to measure the elongation of leaves and shoots (Bovie 1912, 1915, Koningsberger 1922). Through further development, such leaf elongation measurement techniques have become essential tools to understand growth in response to environmental conditions and were partly used in field experiments (Gallagher et al., 1975; Körner and Woodward, 1987; Ben-Haj-Salah and Tardieu, 1995; Christ and Körner 1995, Poiré et al. 2010). More recently, optical approaches based on leaf segmentation in image sequences for monocot leaves or canopies have been developed under controlled conditions (Matos et al., 2014, Poiré et al., 2010; Hartmann et al., 2011) and also for field application (Guo et al. 2013, Grieder et al., 2015). However, these segmentation techniques have not reached the spatial and temporal resolution that is needed in the field to resolve small leaf increments near the thermal limit or diel growth dynamics.

Using artificial marker points either attached to the leaf margins of growing dicot leaves or connected to weight-loaded strings has proven itself effective in allowing precise

video-records of growth rhythms in field experiments (Mielewczik et al. 2013). This technique in combination with a mechanical set-up also appears to be promising in measuring leaf elongation in monocot species. However, it remains to be shown that the method is robust against rain and wind, which is important to measure plant growth under harsh and cold environmental conditions in alpine regions or in lowlands during winter. Furthermore, higher numbers of leaf replicates are needed to enable robust statistical analysis. Developing methods to measure monocot and dicot leaf growth in the field has been one of the technical challenges of this doctoral thesis.

1.5 Hypotheses and aims of the thesis

All experiments were performed under the overarching hypothesis that all cold adapted higher plants face similar temperature related limitations and that similar low temperature thresholds restrict their growth. This hypothesis was developed by Körner (2008), based on similar temperature limitations observed in winter crops and wild, cold adapted plants. Here, I investigate this hypothesis in more detail, aiming at identifying precise elongation rates and relative growth rates of single leaves as well as elongation rates of roots at very low temperatures to depict the absolute low temperature limit for growth. Moreover I aimed at exploring the role of temperature thresholds below 5 °C and test if such thresholds do affect continuous growth rates of leaves and roots. I studied monocot and dicot species to test response differences to temperature and other environmental factors between both groups. I hypothesize that both these taxonomic groups have similar base temperatures for the rate of leaf elongation, leaf expansion or root elongation. Experiments were performed under field conditions to depict the *in situ* situation.

On the physiological level, the hypothesis was tested that the elongation and differentiation of cells into new tissue are most sensitive to low temperature. Moreover, lignification of xylem as a likely thermally sensitive mechanism was tested in response to low temperature in the alpine root growth experiment.

Besides the clarification of thermal growth limits and their physiological explanation, this thesis also aims at contributing to methodological developments to enable the collection of precise growth data close to the thermal limit. The optical marker tracking approach described in Mielewczik et al. (2013) was further developed and applied to a technical set-up for field use to record leaf elongation rates of any kind of monocotyledons. Root growth was also obtained by image sequence analysis using transparent plant

cylinders and the software “SmartRoot” to track the position of root tips (Lobet et al. 2011).

1.6 Chapter overview

Chapter 2

This chapter presents the method called ‘Leaf Length Tracker’ (LLT), which was able to measure leaf elongation rates of all kinds of graminoids. The image-based and weatherproof method was used to collect high-resolution leaf elongation data with considerable sample size in field experiments or under controlled growth chamber conditions. The measurement system was tested on different monocot crop species such as wheat, barley and ryegrass. After first developing the method, it was tested to measure leaf growth close to the low temperature limit in winter wheat and to detect variety specific difference of leaf growth per °C (thermal time) at such cold temperatures conditions. LLT allows the measurement of genotype-specific reactions to any conceivable treatment under in-situ conditions; this method can therefore become an important tool for plant phenotyping and breeding. Moreover, the method can help to bridge the gap between studies performed under controlled laboratory and under field conditions, respectively.

Chapter 3

The temporal dynamics of leaf growth in cold adapted dicotyledons close to limiting low temperatures are to my knowledge, unknown so far. Here, these thermal limits and dynamics were exanimated using winter rapeseed (*Brassica napus*) as dicot model species, grown in the field. Growth of single rapeseed leaves were studied under natural winter field conditions using a marker based image sequence analysis methodology in combination with precise recordings of the environmental conditions at plant height (5 cm), close to the growing leaves. The expansion growth of leaves was calculated as relative leaf growth rates and analyzed by developing a regression model based on the environmental factors, vapor pressure deficit, temperature and light.
Leaf growth was also analyzed to test if circadian rhythms play a role in regulating growth at limiting low temperatures. To determine the absolute low temperature limit of dicot leaf growth, the relative leaf growth rate was correlated with hourly thermal sums above 0 °C per 24 hour time interval.

Chapter 4

This study explores the low temperature limits of tissue formation in alpine plants. A so far unique experimental design was adopted by growing alpine plants outdoors in an alpine setting at 2440 m elevation, but thermostating their root environment. Roots grew against a steep soil temperature gradient, so that the absolute temperature limit of root growth could be identified, and root tip tissue from that physiological barrier was used for histological examinations. Cell sizes, differentiation zones and the degree of xylem lignification were assessed in longitudinal microtome sections.

Additional chapter 5

This publication was written during my PhD time from data collected earlier (not part of this PhD). With this work, I explored root growth of herbaceous alpine species growing under contrasting soil temperatures. Three typical alpine plant species were grown in small containers with root observation windows in a glacier forefield and similar sites, with half of the containers submersed in cold glacial streams. The work revealed clear root growth restrictions at temperatures below 3 °C with the absolute temperature minimum for root growth assumed to be close to 2 °C. Because of the 4-day intervals of the manual census, the thermal constraints and thresholds could not be assessed with certainty, which was the starting point for the PhD project with precisely controlled root temperature and root tip monitoring (Chapter 4).

2 Leaf Length Tracker: A novel approach to analyze leaf elongation close to the thermal limit of growth in the field

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Keywords
phenotyping, plant growth, marker tracking, field conditions, low temperature, monocotyledons, leaf elongation

2.1 Abstract

Leaf growth in monocot crops such as wheat and barley largely follows the daily temperature course, particularly under cold but humid springtime field conditions. Knowledge of the temperature response of leaf extension, particularly variations close to the thermal limit of growth helps defining physiological growth constraints and breeding-related genotypic differences among cultivars. Here, we present a novel method, called "Leaf Length Tracker" (LLT), suitable to measure leaf elongation rates (LER) of cereals and other grasses with high precision and high temporal resolution under field conditions. The method is based on image sequence analysis, using a marker tracking approach to calculate LERs. We applied the LLT to several winter wheat, summer barley and ryegrass varieties, grown in the field and in growth cabinets under controlled conditions. LLT is easy to use and we demonstrate its reliability and precision under changing weather conditions such as temperature, wind and rain. We found that leaf growth stops at a base temperature of 0 °C for all studied species and detected significant genotype-specific differences of LER with rising temperature. The data obtained were found to be statistically robust and were reproducible in the tested environments. Using LLT, we detected subtle differences (sub mm) of leaf growth patterns, which will allow the collection of leaf growth data in a wide range of future field experiments, under different environmental or treatment conditions, studying different graminoid species or varieties.

2.2 Introduction

Leaf growth dynamics in monocot plants are largely determined by temperature, provided soils are moist and fertile, which has been shown in several grass taxa such as wheat, barley, maize and wild grasses (Bliss 1956, Watts 1971, Peacock 1975a, Peacock 1975b, Gallagher and Biscoe 1979, Gallagher et al. 1979, Körner and Woodward 1987, Sadok et al. 2007). The response time of graminoids to temperature is very short and growth rates react within a few minutes to temperature changes and follow the thermal course of their treatment or environmental conditions unless other important abiotic factors, such as water availability and nutrient supply, do not constrain plant growth (Peacock 1975a, Stoddart et al. 1986, Pollock et al. 1990, Walter et al. 2009). However, the sensitivity of plant growth to temperature varies among species. Cold adapted winter cereals or arctic alpine grasses maintain leaf growth under very low temperatures, reaching a limiting temperature (so-called base temperature T_b) close to 0 °C (Gallagher and Biscoe 1979, Gallagher et al. 1979, Körner and Woodward 1987), at the price of lower maximum rates at warm temperatures. Lowland ryegrass has a T_b close to 4 °C (Peacock 1975b) and maize, which originates from warm climates, has a T_b close to 10 °C (Reymond et al. 2003).

The physiological reasons for the strong differences between species with respect to low temperature effects on plant growth are complex. In sensitive monocots such as maize, low temperature diminishes cell production and increases the cell cycle time (Rymen et al. 2007), which contributes to a lower LER. Cold adapted plants maintain cell division until freezing (Körner and Pelaez Menendez-Riedel 1989) and have not been found to be carbon source limited at 5 °C where they still perform 50-70% of their assimilation provision (Körner 2003). Most likely, metabolic processes involving tissue formation and differentiation are most temperature sensitive (Körner 2015) and thus limit leaf elongation.

Above T_b the temperature response of leaf elongation rate (LER) is still less clear than anticipated. For example, in some studies with cold-acclimated winter cereals and ryegrass, the relationship between growth and temperature has been shown to be linear (Gallagher, 1979), whereas an exponential response was reported (Peacock 1975a, Peacock 1975b) in a temperature range between ca. 4 °C and 20 °C.

Others have described the response curve of growth to temperature as a combination of linear and non-linear functions, with a dominating exponential component close above T_b,

a linear component dominating at intermediate temperatures and saturation towards high temperatures (Blum 1986, Voorend et al. 2014).

To increase our understanding of physiological and environmental factors affecting growth, in the field, requires *in situ* non-destructive methods. Subsequent analyses of one and the same organ over and over again is essential, since inter-individual variability of organ size strongly limits the resolution when using destructive methods, even when they are based on a high number of replicates (Walter et al. 2009). Therefore, analyses of leaf area as a proxy of dry weight have been suggested a long time ago (Briggs et al. 1920) and have proved to be a valuable tool in contemporary physiological (Walter et al. 2009) and agronomical studies (Hartmann et al. 2011, Fiorani and Schurr 2013, Furbank and Tester 2011).

Measuring leaf elongation is a precision tool to study plant growth in grasses. Methods have been developed and carried out since the early 20th century using the classical auxanometer approach (e.g. Bovie 1912, Bovie 1915, Koningsberger 1922, Idle 1956, Ranson 1955). With further improvements, these methods have become essential tools to understand growth and the response of plants to their environmental conditions. In monocot plants, a variety of mechanical methods measuring linear extensions or leaf elongation rates have been successfully established also using linear variable displacement transducer (LVDT) or rotary resistance transducer (RRT) techniques (Gallagher et al. 1975, Körner and Woodward 1987, Ben-Haj-Salah and Tardieu 1995, Poiré et al. 2010). More recently, optical approaches based on time lapse imaging of monocot leaves or canopies have been established (Matos et al. 2014, Poiré et al. 2010, Hartmann et al. 2011, Grieder et al. 2015). However, the combination of high temporal resolution and of high throughput in LER analysis in the field has not been achieved with the previous approaches. Such a combined analysis is necessary to uncover more details of leaf growth processes not only at low temperatures, which might represent a major success for plant science and breeding.

In this paper, we present a novel method to measure LER that can be used in the field as well as under controlled conditions. The method is a hybrid between the classical mechanical approach and an imaging-based marker tracking approach described in Mielewczik et al. (2013). Thereby, it combines the advantage of precise elongation analyses with the advantage of an automated, cheap and weatherproof image based recording unit that monitors considerably higher sample sizes of leaves at the same time

with only one measurement unit (camera), and thus, allows the determination of reliable growth rates.

We intended to test whether the setup and approach can; a) be applied in different experimental settings (field and climate chamber) and b) provide enough statistical power to differentiate between LER-temperature response curves under difficult conditions of low temperature.

2.3 Material and methods

2.3.1 Plant material and experiments

Four winter wheat varieties (*Triticum aestivum* L., variety "Combin", "Caphorn", "Cambrena" and "Chaumont"), three distichous spring barley varieties (*Hordeum vulgare* L. f. *distochon*, variety "Ascona", "Eunova" and "Quench") and six rye-grass varieties (*Lolium perenne*) were grown in the following experimental settings.

(1) Winter wheat was cultivated in spring season 2014 in plots of 1.5 x 1.5 m in rows of 17 cm distance at the ETH research station for plant sciences Lindau-Eschikon ("Eschikon", 47.449°N, 8.682°E, 520 m above sea level, soil type: gleyic cambisol, sowing date: 19. October 2013). The plots were part of a larger experiment described in Grieder et al. (2015). Leaf elongation measurements were made with 20 replicates per variety in two consecutive weeks (week 1 & 2) from 25.03.2014 to 07.04.2014 (Fig. 1D).

(2) Spring barley was grown in plots of 1 x 2 m at two contrasting field sites in rows 20 cm apart at Eschikon (sowing date: 7. April 2014) and on a southeast exposed mountainside in Kunkels, Switzerland (46.873°N, 9.409°E, 1180 m above sea level, soil type: calcareous chernozem, sowing date: 10. April 2014). Five weekly measurements were made with seven replicates per variety from 22.04.2014 to 30.05.2014.

(3) Additionally, spring barley was grown in the same period as for the barley field setting in a climate chamber (Conviron, Winnipeg, Canada; sowing date: 4. April 2014) in pots (10 x 10 x 20 cm, nine plants per pot) filled with a 4:1 mixture of soil (Landerde, Ricoter, Aarberg, Switzerland) and silica sand (0.5 – 2 mm). Illumination cycle was comparable to the field conditions with a day/night period of 13/11 h reaching a light intensity of 275 μmol photosynthetically active radiation (PAR) m^{-2} s^{-1}. The average day/night temperature was 10/2 °C, respectively, and relative humidity was kept at 60±15%. Measurements were made with seven replicates per variety from 17.04.2014 to 20.05.2014.

(4) Ryegrass was grown in a climate chamber of the same type as described above to test the method on a monocot plant that is not a cereal crop. Plants were cultivated in a commercial potting mix substrate ('Spezialmischung 209', RICOTER Erdaufbereitung AG, Aarberg, Switzerland) with a light/dark photoperiod of 16/8 h. The average day/night temperature was 25/15 °C, and relative humidity was kept at 50±15%. We tested five genotypes with each four replicates and measured leaf elongation for four days.

All investigated field plants were fertilised during the measurement period with 40 kg N ha^{-1} (Landor Nitrate, 27% N +2.5% Mg, Landor, Birsfelden, Swizerland), 40 kg P_2O_5 ha^{-1} and 80 kg K_2O ha^{-1} (Agroline Concentro, 13% N, 13% P_2O_5, 26% K_2O, Agroline AG, Roggwil, Swizerland) and were watered when necessary to exclude any confounding effect on plant growth except temperature.

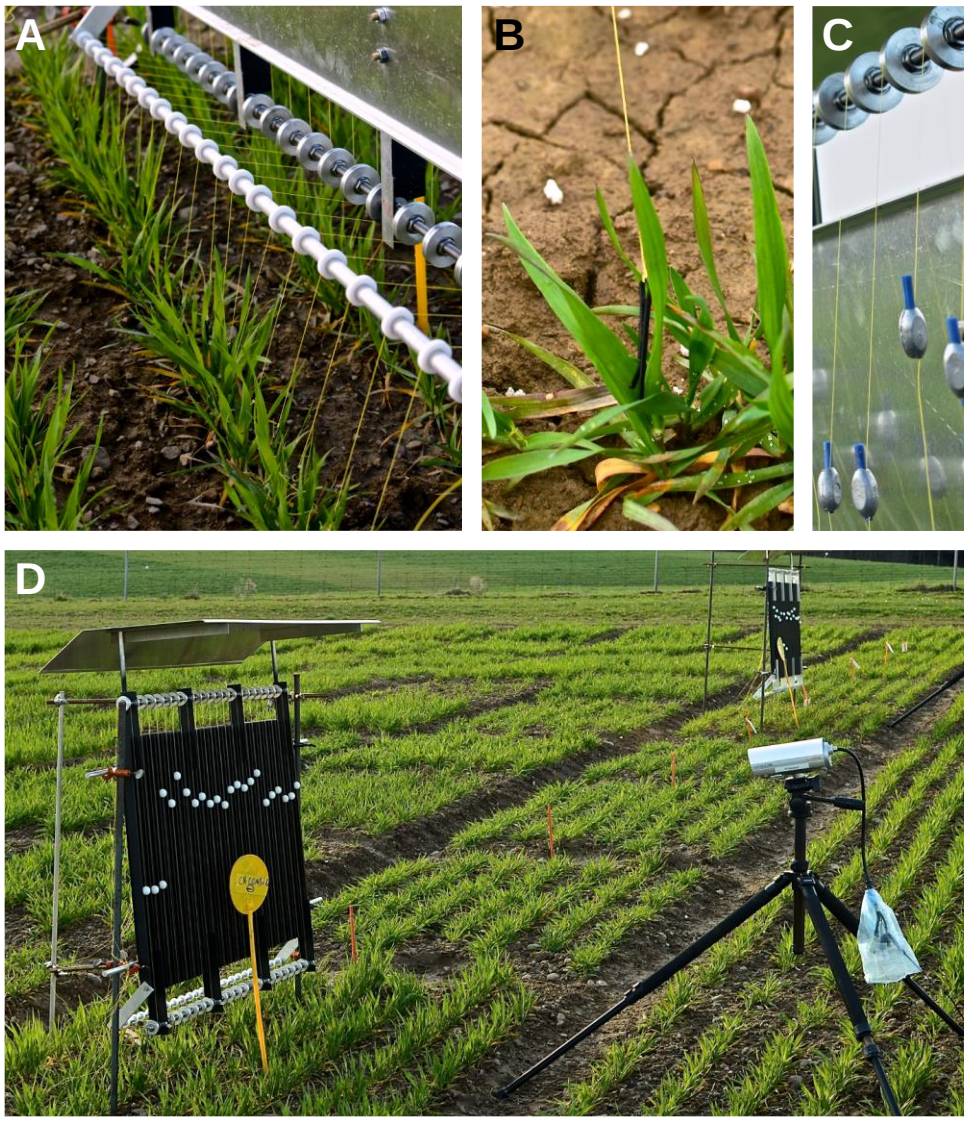
2.3.2 Experimental set up of the leaf elongation measurements

For each investigated plant of a given species or genotype, we chose one new emerging leaf at the youngest measurable developmental stage and connected the leaf tip with a small hairpin (25 mm length, Fig. 2.1 B) to a 150 cm long thread (Dyneema® fibers, ø 0.16 mm, tensibility 1%, Climax, Ockert GmbH**,** Puchheim, Germany). The thread was guided through a plastic reverse roller to the black aluminum measurement panel (100 x 60 cm). On the panel, the thread was guided by another two ball bearing mounted reverse rollers (35 mm precision miniature bearing, Sapporo Precision Inc., Sapporo, Japan). The first roller guided the thread into the vertical plane of the panel and the second roller tensed the thread with a 20 g counterweight in the back of the panel (Commercial weights used by anglers, Fig. 2.1 A, C, D). A white bead (Polyoxymethylene®, 6 g, ø 20 mm, Maagtechnic, Füllinsdorf, Switzerland) was connected to the twine, which was able to move up and downwards on the vertical plane of the panel (Fig. 2.1 D), guided by an aluminum u-rail (internal dimension 19 mm). The panel was tilted by 5° backwards to force the beads to move along the u-rails. With leaf elongation, the bead moved upwards, drawn by the 20 g counterweight on the back of the panel that was balanced by 6 g weight of the bead and 1 g roller resistance, resulting in 0.13 N tensile force exerted on the extending leaf. This tensile force was sufficient to gently stretch a leaf into the vertical plane, avoiding confounding effects by rain and wind and has shown to leave natural leaf elongation unaffected (Gallagher 1976, Sadok et al 2007, Walter et al 2002). We built four panels, each providing 23 u-rail measurement positions from which three were used as reference positions (not connected to leaves). The panels were attached to two iron rods (2 m, ø 2 cm) that were inserted ca. 40 cm into the soil (field) or that were clamped to the testing table (climate chamber). In the field, panels were stabilized by two additional iron bars/poles to minimize wind movements (Fig. 2.1 D).

Digital images (grey scale) were collected with a tripod mounted LupusNET HD camera (LUPUS-Electronics, Landau, Germany; 1920 x 1080 pixels (2.1 pixel mm^{-1}), which was positioned at a distance of 2 m from the panel to monitor the white beads every 120 s. During night, images were illuminated by near-infrared diodes in the camera. Zoom and focus of the camera were adjusted to fit the 100 x 60 cm dimensions of the panel.

In field experiments, we recorded temperature and weather data with a Hobo weather station (Onset Computer Corporation, Bourne, MA, USA). Air temperature was measured in 2 m and at 5 cm above soil level ("plant height"), soil temperature at 1 cm and 10 cm soil depth (°C). Further more, precipitation (mm), relative humidity (%), volumetric soil water content (Vol.%), photosynthetically active radiation (PAR) and wind speed ($m\ s^{-1}$) were recorded. In the climate chamber experiment with barley, air temperature at plant height was measured by small temperature sensors (Onset Computer Corporation, Bourne, MA, USA) and room temperature, relative humidity and radiation (PAR) was logged by the climate chamber. In the climate chamber experiment with ryegrass we measured meristem temperatures instead of air temperature at plant height with six type T needle thermocouples (ø 0.1 mm; Omega, Stamford, CT, USA) and recorded data with a Campbell CR10X data logger (Campbell Scientific, Logan, UT, USA).

Figure 2.1



Experimental set up of the measurement panel in the field. Similar panels without a roof were used in climate cambers. (A) Row wise measurement of 20 wheat leaf replicates. Here, it is visible how threads are passing the first and second reverse rollers. (B) Close up view of a leaf tip attached by a hairpin. (C) 20 g counterweight and third reverse roller. (D) Wheat micro plots and the installed measurement panels with white beads and a near-infrared camera in front. The first three beads on the left are used for reference measurements. The panel was north facing to avoid shading of the investigated plants. The panel roof was installed to prevent confounding effects by raindrops and snow on the beads.

2.3.3 Image processing

To extract leaf elongation rates from the image sequences, we developed a software in Matlab 8.2 (The Mathworks, Natick, MA, USA) called "Leaf Length Tracker" (LLT). The program can be operated by a simple graphical user interface (detailed instructions in supplementary material, Fig. S2.1) and consists of three central parts: Marker (bead) tracking, correction of lens distortion (rectification) and displacement determination.

To calculate the image positions of the beads, which we used as indirect artificial landmarks for leaf elongation, the marker tracking approach described in Mielewczik et al. (2013) was applied. The algorithm is based on a cross-correlation algorithm with position interpolation for sub pixel accuracy. After loading an image sequence into the tracking software, bead positions and surrounding search areas were set manually using the interface (Fig. S1, software manual) and the position estimation is started for each bead, which is tracked throughout the image sequence of an experimental period (for a detailed description of the marker tracking algorithm see: Mielewczik et al. 2013). To ensure a high quality template position (bead) tracking, a bead was considered to be lost when the cross-correlation coefficient (quality of position localization) was below 0.5, which would result in unreliable results (typical values were: 0.85-0.95, see results). When losing a tracking position, the software tries to localize the bead in the next image of the sequence and deletes the displacement data of the particular bead in the problematic image (see software manual, supplementary material, Fig. S2.1).

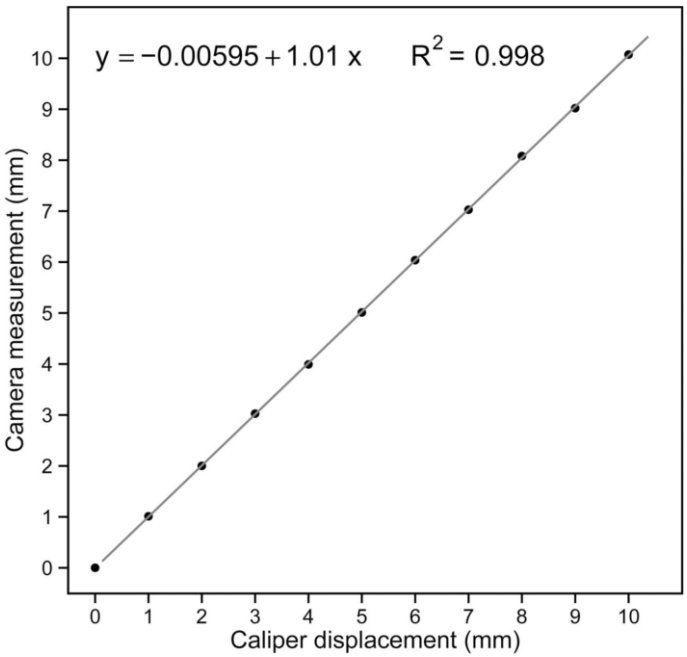
To compensate for lens distortion effects, we took a calibration image of a checkerboard (square size 45.5 mm) of the size of the measurement panel before each experiment, which was positioned in the plane of the beads. The transformation parameters for rectification of the image were calculated and then applied to the pixel coordinates of the tracked beads, which resulted in bead positions in mm. By giving the size of the checkerboard squares, an accurate pixel size was determined for the rectified images and therefore for the corrected bead positions. For extraction of displacements of the bead positions, a second order polynomial was fitted to all positions of each bead, on which all positions were projected. Bead displacements were measured as distances along the polynomial projection in millimeters throughout the image sequence.

Leaf Length Tracker can be downloaded at SourceForge (https://sourceforge.net/projects/leaf-length-tracker/) and is compiled for Windows (64-bit). A user manual to operate the program is provided in supplementary material (Fig. S2.1). Leaf Length Tracker requires the Matlab Compiler Runtime: MCR; Vers. R2013b (8.2 64-Bit, The Mathworks, Natick, MA, USA) to be installed on the user machine (download at http://www.mathworks.com/products/compiler/mcr/).

2.3.4 Verification of image based displacement measurements

We verified the accuracy of our measurement set up at each of the 23 possible panel positions by manually moving the single measurement units (hairpin, twine, 20 mm bead and 20 g counterweight) in steps of 10 x 1 mm using a digital caliper (Series 500, Mitutoyo, Kawasaki, Japan; accuracy ±0.02 mm). This was done under open-air conditions next to the Eschikon field site. We recorded the 1 mm displacement steps of the beads with the camera and correlated the image-derived results against the manual measurements (Fig. 2.2).

Figure 2.2



Linear correlation of image based displacement measurement against the manual caliper controlled movement of a bead. Similar correlations were found at each of the 23 panel positions.

2.3.5. Leaf elongation calculation and statistics

Leaf elongation rates (LER) were calculated per hourly intervals (mm h^{-1}) as the ratios of leaf extension (ΔL, mm) per unit time (Δt, h) using Eq. 2.1:

$$\text{Eq. 2.1: } LER = \Delta L/\Delta t$$

To correct LER for thread stretching by moisture and temperature or wind induced movements of the panel, three reference measurements were taken throughout each measurement period by attaching the threads not to a leaf but to a ground nail, inserted into the soil. The mean displacements of the beads attached to those threads were subtracted from the measurements, which further improved the accuracy of the procedure (supplementary material, Fig. S2.2).

For graphics and statistics we averaged all temperature and weather data according to the LER intervals. We used simple linear regressions to describe the relation of leaf growth and temperature. Here, air temperature at plant height (5 cm above ground, in field experiments) and temperature in the meristem (ryegrass experiment in climate chamber) explained leaf elongation better (higher R^2-values, Fig. S3) as air temperature 2 m above ground or soil temperature (5 cm below ground).

For winter wheat, we corrected LER for thermal time by using Eq. 2.2 as described in Reymond et al. (2003, not considering vapor pressure deficit) to test the linearity of the genotypic specific temperature response.

$$\text{Eq. 2.2 } LER = i + aT$$

where a is the linear coefficient between temperature and LER, and T temperature (°C) with the intercept fitted through i. We set i = 0 °C, as our studied species marginally (max. ± 0.26 °C) deviated from this basis temperature (T_b = 0 °C, see results).
For thermal time correction we calculated a (mm h^{-1} $°C^{-1}$) using mean LER (fitted through zero) per variety and subtracted each observed single leaf LER.

To account for the genotypic response of LER to temperature we calculated a (mm h^{-1} $°C^{-1}$) using Eq. 2 for each measured leaf in all experiments. To test for genotypic differences between a a one-way analysis of variance (ANOVA) was conducted and genotypes were grouped by posterior testing using Tukey-Kramer HSD test, for each measurement period (week).

All growth rate calculations, statistical analyses and diagrams were performed using R Statistical Software (version 3.0.2; R Core Team 2014) and the packages “ggplot2” (Wickham 2009) and “gdata” (Warnes et al. 2014).

2.4 Results

2.4.1 Method functionality and accuracy

Based on prior experience we found that white beads of 20 mm diameter on a black background (100 x 60 cm) were well suited for automated marker tracking throughout an image sequence. The high grey value contrast (white/black) enabled measurements under all light conditions in the field such as bright sunshine or fluctuating weather with transient shading by clouds. Due to the solid construction of the aluminum panel, wind gusts up to 16 m s^{-1} did not affect our measurements (Fig. S4). Our four measurement panels functioned reliably and automated data collection for up to seven days was possible in the growth chamber and in the field: even at the remote mountain study site in Kunkels (1180 m a.s.l.).

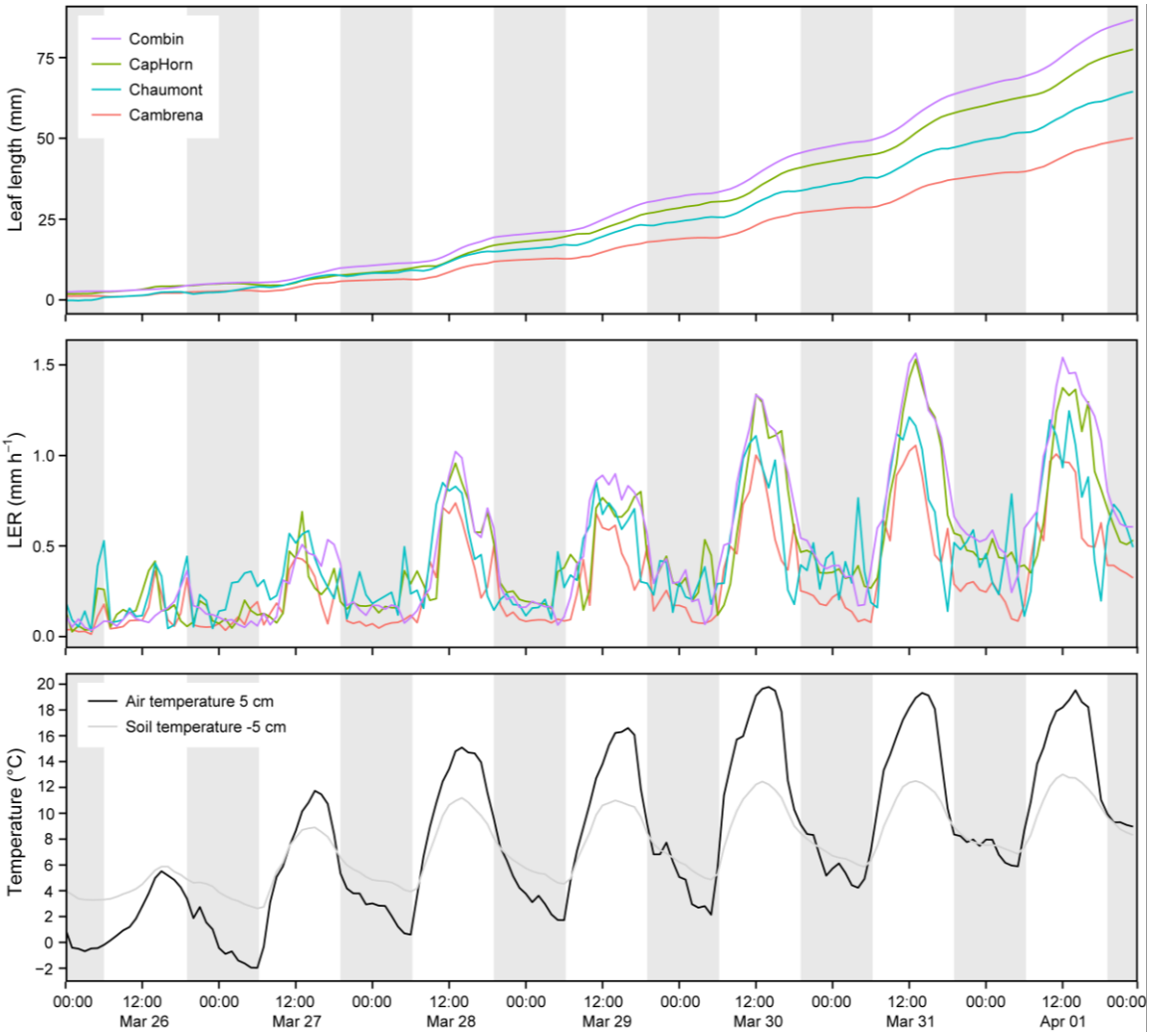
Application of LLT was straight forward and time efficient. The setup of one measurement panel, the attachment of 20 leaves and the installation of the camera took less than one hour. The subsequent software analysis of an image sequence of 5000 pictures required ca. 40 min using a standard personal computer (Intel® Core™ i5 processor with 3.33 GHz CPU and 4.0 GB RAM). The cross-correlation coefficient (CC) of the bead position tracking was >0.95 in daytime images and >0.85 in the night. Bead position loss due to poor CC <0.5 was observed rarely, mostly during bad weather conditions.

We tested the accuracy of our system by comparing, manual, 1.0 mm stepwise displacement of the beads and image based measurements and found the correlation between these was >0.998 (R^2) for each of the 23 bead positions (Fig. 2.2). When subtracting the image based results each by 1 mm (1.0 mm caliper controlled movements), the mean error of all 230 camera-derived measurements (10 measurements x 23 bead positions on the panel) was 0.029 mm. We also checked the displacement of the fixed (non-moving) reference beads during these measurements and found they deviated on average 0.012 mm, which we consider as the technical resolution limit of our method.

2.4.2 Leaf growth and temperature

We assessed the functionality of LLT by monitoring wheat and barley leaves at Eschikon and Kunkels in the field. The leaves of wheat and barley needed ca. five to seven days to reach their final size under the prevailing low springtime temperatures in the field and in the barley climate chamber experiment. LERs followed precisely the daily temperature course (as shown for a representative period in Fig. 2.3). In winter wheat, LER was below 0.25 mm h^{-1} with a few peaks up to 0.5 mm h^{-1} at temperatures below 5 °C and consequently grew less than 5 mm within the first three days. As soon as the air temperature, at 5 cm height, rose above ca. 5 °C LER started to increase abruptly above 0.5 mm h^{-1}. With more pronounced diurnal temperature fluctuations, we found genotype-specific responses to temperature that became greater the higher the temperature rose (last three days in Fig. 2.3). Variety “Cambrena” was most sensitive to low temperatures in week 1 and had the lowest growth rates during day and night. When temperature was increasing after night, “Chaumont” and “Caphorn” showed an earlier increase of LER. After the daily temperature maximum was reached, “Combin” and “Caphorn” retained high LER longer than “Chaumont” and “Cambrena” (Fig. 2.3).

Figure 2.3



Time series of "week 1" of leaf length measurements in four wheat varieties from March 26th until April 2nd 2014. Measurements were taken from leaf 7, when first emerged, until the leaves were fully developed. The upper graph is the mean cumulative leaf length of n = 20 leaves per wheat variety. The middle graph shows the corresponding mean LER. The lower graph shows the corresponding air temperature measured at 5 cm above ground and the soil temperature measured 5 cm below ground. Grey stripes indicate night hours.

Simple linear regressions of mean LER per wheat variety and air temperature resulted in R^2 >0.61 (Fig. 2.4). The response of LER to temperature was different in week 1 and 2 depending on the temperature range the leaves were exposed to. In "week 1" with temperatures from -2 to 20 °C, "Combin" was the most vigorously growing variety whereas "Cambrena" was the most sensitive to low temperature (Fig. 2.3 & 2.4). In week 2, when temperatures were always above 5 °C, "Cambrena" and particularly "Chaumont" had a higher LER while "Caphorn" did not profit from higher temperatures. The four wheat varieties showed also differences when correcting LER for thermal time (Fig. 2.4, LER - *a*T). The thermal time model showed a good fit for "Combin" and "Cambrena" in week one, which indicates a linear temperature response (high peak in histograms of Fig. 2.4). However, the model underestimated *a*, the linear growth per mm h^{-1} $°C^{-1}$, of the other two varieties in week 1 at temperatures >5 °C and all three varieties in week 2 at temperatures <15 °C, which is also indicated by the small shifts to the left or right from the normal distribution in the histograms (Fig. 2.4).

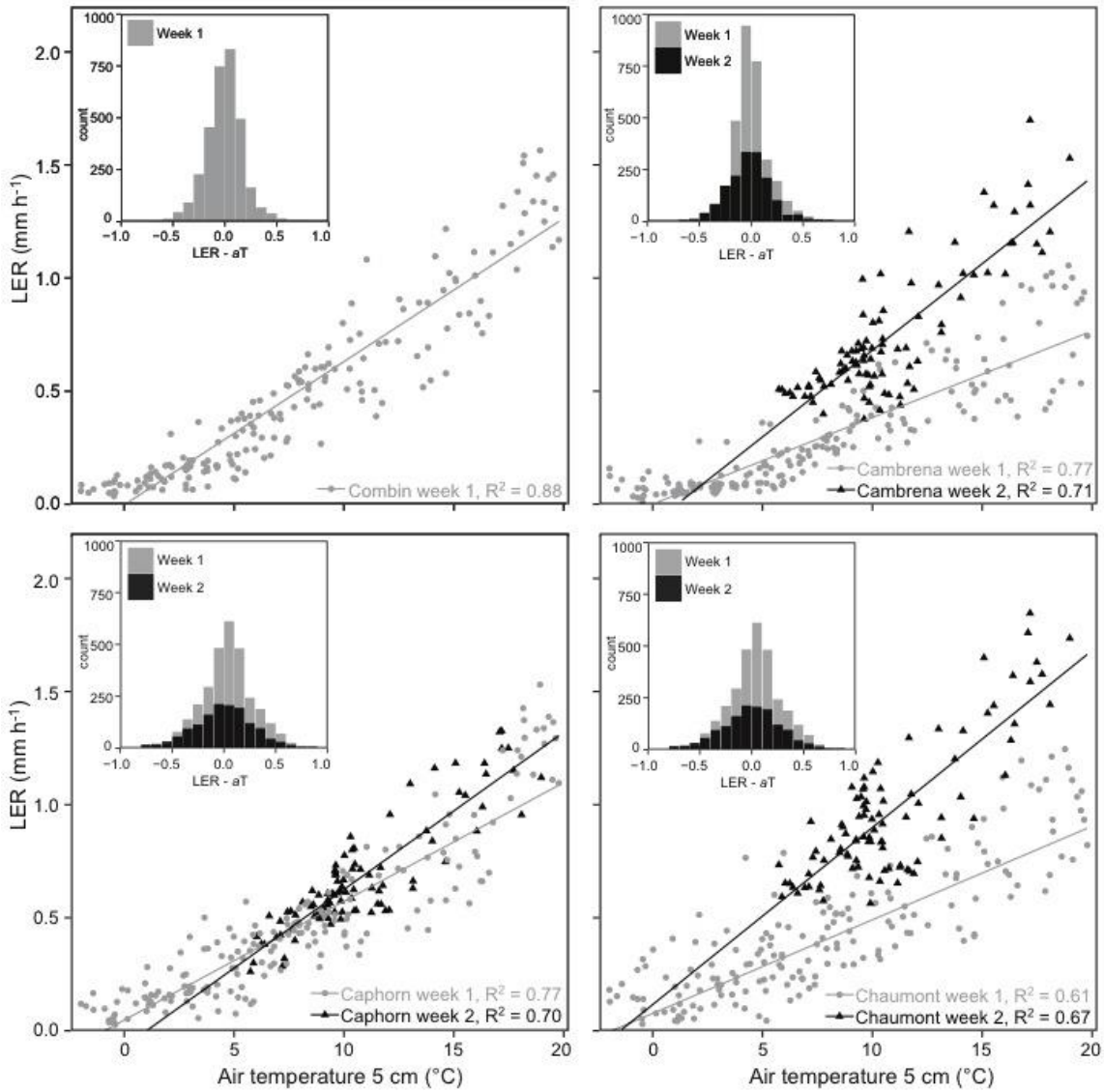
When testing the *a* of single leaf linear correlations we found the genotypic temperature responses previously shown in Figure 2.4 to be significant within week 1 & 2 (ANOVA, P <0.001, supplemetal material, Fig. S2.4), which did result in significantly higher or lower *a* between genotypes in both measurement periods (different letter in supplementary material, Fig. S2.5, Tukey-HSD). We could also identify genotype-specific growth response to temperature in summer barley grown in the field as well as in the climate chamber (ANOVA, P <0.01, Fig. 2.5). The variety "Ascona" always showed the highest *a* independent of temperature range or the field location; while "Quench" and "Eunova" were grew slower (*a*) and were comparable with each other. "Quench" showed somewhat higher growth rates at low temperatures compared to "Eunova" and vice versa at higher temperatures (Climate chamber Fig. 2.5). This pattern was almost consistent in the two contrasting field sites. Furthermore, results from the two field sites were replicated in the climate chamber for a very low temperature range (2 – 8 °C), in which "Ascona" showed highest LER followed by "Quench" and "Eunova" (Fig. 2.5). Significant genotypic-specific responses in barley were found when temperature was very low or the temperature range was big enough (ca. 14 K, Fig. 2.5).

In ryegrass, we found a trend for genotype-specific reaction of LER to temperature in the five varieties, which were grown at a higher temperature range (15 – 25 °C, supplementary material, Fig. S2.6). However, this was not significant, when assessed by ANOVA (P = 0.29). Linear correlations using meristem temperatures resulted in higher R^2

values compared to correlations using the room temperature of the climate chamber. Furthermore, room temperature of the climate chamber was slightly higher than meristem temperatures (supplementary material, Fig. S2.3).

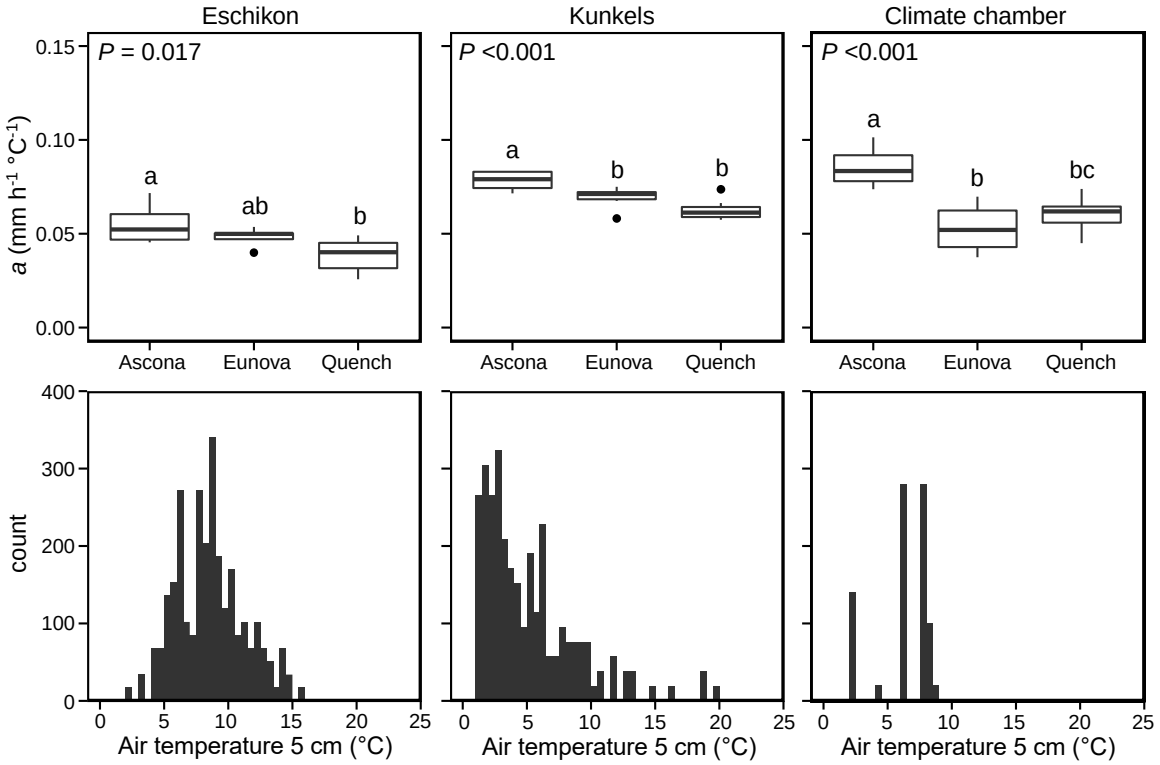
The x-intercepts of LER plotted against temperature were close to 0 °C for all studied species and periods when applying normal linear correlations without fitting the intercept through 0 (as shown for wheat in Fig. 2.4). There was no obvious trend among species (wheat: 0.02 ± 0.15; barley: -0.15 ± 0.26; ryegrass 0.18 ± 0.22 °C) or among genotypes in the specific periods (supplementary material, Fig. S2.5, S2.6 and S2.7).

Figure 2.4



Linear correlations of mean LER (each n = 20 leaves) and air temperature at 5 cm above ground for each of the four wheat varieties. Grey dots refer to "week 1" and black triangles to "week 2" (Leaf 8) of the experiment. The histograms in upper left corners show LER corrected for thermal time (LER - *a*T) for "week 1 & 2". Note: there is no data for "week 2" for variety "Combin".

Figure 2.5



Upper row: LER per °C (*a*) of three summer barley varieties (each n = 7 leaves) from the two field sites and the climate chamber. *P*-values are derived from ANOVA and letters above boxes indicate significant genotype-specific differences (Tukey-HSD, $P < 0.05$). Lower row: Histograms of mean temperature per hour (in steps of 0.5 °K) from the period of measurements.

2.5 Discussion

LLT has proven to be a precise method to study leaf elongation rates of monocot plants and is particularly suited for field experiments. The measurement panel and image acquisition worked reliably in all four experiments. The software analysis ran robustly and automatically, once an image sequence was loaded and the bead positions and search area were set. Software problems in prior attempts such as a loss of the tracking regions during changing weather conditions were overcome by the colour contrast (white beads/black background) and bead tracking resulted in high cross-correlation-coefficient values (>0.85), which ensured reliable measurements.

Leaf elongation instantly caused the movement of beads due to the small rolling resistance of the three reverse rollers. The tensile force exerted on the leaf added up to 0.13 N, corresponding to a virtual, attached weight of 13 g. The aluminum u-rails prevented any shaking of the beads and allowed up or downward oriented movements only. The sensitivity of our method allowed us to record very small LERs at temperatures below 5 °C of ca. 0.5 mm h^{-1} as well as a fast increase of up to 1.5 mm h^{-1} when temperatures rose during the day (Fig. 2.3). A measurement error of 0.03 mm is small considering the error of the caliper (0.02 mm), which we used to move the beads in steps of 1.0 mm. We therefore carefully interpret this error since we can not tell whether errors <0.02 mm are due to the caliper precision limit or even more probably, due to operator error during bead movement. However, when evaluating the non-moving reference beads during verification measurements we arrive at a mean displacement of 0.012 mm. Here, the software algorithm tries to calculate the displacement from the previous position, thus the resulting error is the true image based resolution limit since these beads did not move. Yet, even the limit of 0.03 mm should allow distinguishing, whether or not a leaf has grown in a 2 minute time interval at temperatures >5 °C, in which it has exceeded this ‘minimum distance’ at least by a factor of two. Furthermore, the calculated LERs per hour are very small and positive at low temperatures (first two days in Fig. 2.3), which would not have happened if we had recorded only error noise of ± 0.03 mm.

LERs below 0.25 mm h^{-1} resulted only in minor leaf length increment and thus biomass accumulation is negligible (first three days of Fig. 4). We cannot explore whether those small movements are elastic, reversible elongations due to a transient fluctuation in turgor or whether they are connected to underlying permanent cell elongation.

For all three species, winter wheat, summer barley and ryegrass we found T_b for leaf elongation close to 0 °C with minor differences between species and genotypes (supplementary material, Fig. S2.5, S2.6, S2.7). These thermal limits are in accordance to the literature for wheat and barley, which are reported to be between 0 and 1 °C (Gallagher et al., 1979; Gallagher and Biscoe, 1979; for a review see: Porter and Gawith, 1999) but lower for ryegrass, for which a basis temperature of ca. 4 °C was described before (Peacock, 1975a).

Our growth rates for wheat, barley and ryegrass (between 0.5 and 2.5 mm h^{-1}) are similar to LERs that were obtained using other methods such as LVDTs or manual ruler measurements (Gallagher et al., 1976; Peacock, 1975a) under comparable growth conditions in a temperature range of 5 to 25 °C. However, LLT enables highly temporally resolved and precise data collection over periods of seven days without interruption. This allows observation of LER close to the thermal limit of growth (T_b), which we were interested in. This allows detecting short term and genotype-specific reactions of LER to temperature, which were found to be significant in wheat and barley in the field and the climate chamber (Fig. 2.4 & 2.5).

The temperature regime the leaves were exposed to had a big influence on the growth performance of the specific genotypes. Two wheat varieties “Cambrena” and “Chaumont” had a higher *a* at the temperature range >5 °C of week 2 while “Caphorn” did not profit from higher temperatures but grew better in week 1 when temperatures were mostly below 10 °C (Fig. 2.4). Similarly, the barley variety “Eunova” grew faster at higher temperatures, while “Quench” did not increase LER as much as “Eunova” or “Ascona, which always grew faster independent of the temperature range. These specific reactions to temperature underline the potential of different genotypic material regarding growth.

In this paper we applied linear correlations to explain growth in relation to temperature, which worked very well in the temperature range of 5-15 °C. However, our data suggest also a non linear component in the lower temperature range below 5 °C (wheat varieties “Combin” and “Cambrena”, Fig. 4) and above 15 °C, where LER was higher than expected by the linear model, with the majority of data points situated above the regression line in both cases (Fig. 2.4). Small shifts of the peak from normal distribution (LER $-aT$, histograms in Fig. 2.4) support a non-linear influence. Deviations from the thermal time model are again species specific since varieties “Caphorn” and “Chaumont” showed a linear response at low temperature.

LLT can be used to record LER data with a single recording unit (camera, panel and beads), which produces statistically robust results when measuring 20 leaves per crop variety (winter wheat data) or allows fast screening of genotypes when measuring 6 x 3 leaves per variety (barley experiment) or 4 x 5 leaves (ryegrass experiment). Simultaneous measurement for hundreds of plants is easily in reach, as the described setup is inexpensive opening the possibility to apply multiple measurement panels or even double the number of measurement position per unit. Particularly, monitoring growth throughout the entire development course, *in situ,* of the studied leaves to our knowledge has not been achieved under field conditions so far.

Our method advances the state of the art in plant growth measurements and allows corroboration between controlled laboratory environment and field, which is a recognized gap in non-destructive growth monitoring (Dhondt et al., 2013; Araus and Cairns, 2014; Nelissen et al., 2014; Wuyts et al., 2015). Testing plant environment interactions with regard to molecular traits or rising climatic stress factors such as temperature or drought is an important tool for future plant breeding (Araus and Cairns, 2014; Grieder et al., 2015) and will contribute to the basic understanding of plant physiology. Our method might also be useful to study other graminoids and many other treatment factors.

2.6 Supplementary material

Two additionally files are provided in the supplementary material of this thesis. The first is Figure S2.1, a step-by-step manual, which guides users trough the software analysis of an image sequences. The second file contains Figure S2.2 – S2.7. Figure S2.2: The result of reference bead subtraction. Figure S2.3: Linear correlations of LER to different temperature measurements. Figure S2.4: Linear correlation of LER and wind gust speed. Figure S2.5: Boxplots of significant genotypic-specific leaf growth per °C in winter wheat, x-intercepts and temperature histograms. Figure S2.6: Boxplots of ryegrass leaf growth per °C, x-intercepts and temperature histogram. Figure S2.7: X-intercepts of summer barley from linear correlations of LER and temperature.

2.7 Acknowledgements

The authors thank Christoph Grieder for providing the winter wheat plant material and for help in developing the R codes for data analysis, Kristina Jonaviciene for providing the ryegrass material, Andreas Bruun, Johannes Hunkeler and Michael Friedli who helped developing previous setups of the method and Christian Körner, Johanna E. Wegener and two anonymous reviewers for valuable comments to an earlier manuscript draft. Especially, we would like to thank Werner Nagelmüller for fruitful ideas and technical realization of the LLT panel.

3 Diel leaf growth of rapeseed at critically low temperature under winter field conditions

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Keywords
plant growth, dicots, winter, leaf expansion, diel growth rhythm, vapor pressure deficit, modeling, marker tracking, image sequence analysis

3.1 Abstract

Growth and development of winter crops is strongly limited by low temperature during winter. Monitoring the temporal dynamics and thermal limits of leaf growth in that period can give important insights into the growth physiology at low temperature, crop management and future breeding traits for winter crops. In this study, we focused on winter rapeseed as a model, dicotyledonous winter crop to study leaf growth under natural winter field conditions. Leaf growth was measured using a high-resolution marker based image sequence analysis method and the results were evaluated in the context of environmental conditions. Leaves stopped growing at a base temperature of 0 °C. Above ca. 4 °C, leaves grew with a diel (24 h) growth rhythm, which is typically known for dicots at thermally non-limiting growth conditions. Relative leaf growth rates at temperatures above this 4 °C threshold were higher at night and showed a pronounced depression during the day, which we could describe by a model based on the environmental factors vapor pressure deficit (VPD), temperature and light with VPD exerting the strongest negative effect on leaf growth. We conclude that leaf growth of the selected model species at low temperatures shows a transition between pronounced environmental regulation and a superposition of environmental and internal, possibly circadian-clock-dependent regulation.

3.2 Introduction

Low temperature plays a critical role in the growth cycle of both dicot and monocot winter crops such as rapeseed (*Brassica napus*) and winter wheat (*Triticum aestivum*). Besides the importance of low temperatures for vernalization (Chouard 1960) and the development of freezing resistance (Kacperska & Kulesza 1987, Rapacz 1998, Sakai and Larcher 2012), it restricts leaf growth and thus defines the duration of the growing season for cold temperate plants such as winter crops. From an agronomical perspective, the 'growing period' is most commonly defined as the day after the daily mean temperature has exceeded 5 °C for five days in a row and ends when temperatures fall below this threshold (Carter 1998, Frich et al. 2002). Alternatively, other definitions of the growing period have used the last and first day of frost (Skaggs & Baker 1985, Robeson 2002). Periods outside this definition are designated as the time of vegetative dormancy. However, winter crops do grow during this cold period of the year, as demonstrated by increasing biomass, leaf emergence, leaf length increments or increasing canopy cover (Mendham et al. 1981, Slafer & Savin 1991, Körner 2008, Grieder et al. 2015).

In case of monocotyledonous, graminoid plants, leaf growth close to the lower thermal limit is relatively well characterized. Leaves grow whenever the temperature is above a species-specific base temperature between 0 and 1 °C and the temporal pattern of growth is correlated with the daily temperature fluctuations (Gallagher and Biscoe 1979, Gallagher et al. 1979, Porter & Gawith 1999, Nagelmüller et al. 2016). The growth of cold adapted dicots close to the thermal limit is less clear. Simple ruler measurements of single leaves in sugar beet and rapeseed with a daily or weekly frequency indicated a minimum temperature for leaf increment close to 5 °C (Milford et al. 1985, Rapacz et al. 2001, Körner 2008). Yet, the 'diel' leaf growth variation during 24 h in a fluctuating low temperature field environment remained open. It is unknown, whether dicot leaves show a diel growth pattern also at low temperatures as it is known from studies with thermally non limiting growth conditions (as reviewed in Walter et al. 2009). At these higher temperatures, dicot leaves typically show peak growth during the night. Thus, leaf growth in dicots is thought to be regulated not only by temperature but also by the interaction of environmental factors with endogenous rhythms such as the circadian clock (Walter et al. 2009, Ruts et al. 2012). It remains to be tested if these factors control leaf growth at low temperature.

In dicots, the expanding leaf tissue is not sheltered by the leaf sheath compared to the expanding leaf zone in leaves of monocot, graminoid species and is much more exposed to fluctuating environmental conditions such as light, temperature and relative humidity of the air. Most prominently, fluctuations of vapor pressure deficit with peak values at daytime can limit expansion of leaf tissue. This is due to excessive evapotranspiration and loss of turgor pressure even under well-watered conditions (Ben Haj Salah and Tardieu 1996, Tardieu et al. 1999).

Here, we are aiming to explore how leaf growth in dicots is regulated under cold winter conditions and test, how leaf growth is regulated, besides by temperature, by other environmental factors such as VPD or by endogenous rhythms. Moreover, it is tested, whether the absolute low temperature limit for leaf growth in dicots is comparable to that in monocots. We used winter rapeseed as a model species since it is agronomically the most important dicot winter crop and the species is well established for studying plant physiology in the cold (Stefanowska et al. 1999). Plants were grown in the field and the expansion growth of leaves was measured during December and January using an image based time series analysis method (Mielewczik et al. 2013). Such continuous high-resolution measurements in single leaves have shown to be appropriate to study short-term effects of leaf growth in dicots (Walter et al. 2009, Mielewczik et al. 2013). However, such measurements were not done at low temperatures directly in the field and even with regular freezing of the leaf tissue.

3.3 Materials and methods

3.3.1 Experimental set up

Winter oilseed rape (*Brassica napus*, L. var. *oleifera* f. *anua*; variety Avatar, Ufa, Fenaco, Switzerland) was grown in a plot of 2 x 4 m at the ETH research station for plant sciences Lindau-Eschikon (Fig. 1; 'Eschikon'; 47.449° N, 8.682° E, 520 m above sea level; soil type: gleyic cambisol; sowing date: 29 August, 2014). After germination, the number of plants was reduced to 25 individuals per square meter to generate enough space for the leaf growth measurements. Plants were fertilized twice before the measurement period with 40 kg N ha^{-1} (Landor Nitrate, 27% N +2.5% Mg; Landor, Birsfelden, Switzerland), 40 kg P_2O_5 ha^{-1}, and 80 kg K_2O ha^{-1} (Agroline Concentro, 13% N, 13% P_2O_5, 26% K_2O; Agroline AG, Roggwil, Switzerland) to exclude nutrient deficiency as growth restricting factor.

Leaf growth was monitored in the rosette stage of the study plants from 14 December until the 17 January. Leaf growth was measured in single leaves using a marker tracking method based on image sequence analysis, called "Martrack Leaf" (Mielewczik et al. 2013). Young, similar sized, newly unfolded leaves of eight replicate plants were selected and growth was monitored for 5 to 6 days. After this first study period, new young leaves were selected for growth monitoring. This procedure was repeated for four study periods. In each measurement period, leaves were separately fixed in the lower level of double story round metal frames by using five strings that were glued to the leaf margin (Fig. 1). To provide artificial landmarks for marker tracking, five small white beads (Polyoxymethylene®, 0.5 g, ø 6 mm, Maagtechnic, Füllinsdorf, Switzerland) were threaded onto the strings, which were fixed to the margin of the leaf. The strings were threaded over the lower and upper levels of the round metal frame. The leaves were carefully tensed in the horizontal plane with the force of 10 g weights attached to each end of the five strings (Fig. 3.1). The force of in total 50 g was enough to prevent leaves from moving induced by wind or natural leaf movements. Digital images (grey scale) of the leaf and the marker beads were recorded in 120 s time intervals with weatherproof Lupusnet HD cameras [1920×1080 pixels (2.1 pixel mm^{-1}); Lupus-Electronics, Landau, Germany], which were mounted ca. 40 cm above the measured leaves using iron rods that were drilled into the soil (Fig 3.1). Nocturnal images were illuminated by camera-internal near-infrared diodes. The image sequence of each studied leaf was processed with the "Martrack Leaf" software resulting in pixel area values per 120 second time interval (for details see Mielewczik et al.

2013). Due to the loss of marker tracking beads caused by the harsh winter environment, the number of leaf replicates for growth analysis was reduced to five leaves in study period one, two and four and to six leaves in study period three.

Temperature and weather conditions were recorded every 10 min by a Campbell CR1000 data logger (Campbell Scientific, Logan, UT, USA). Air temperature (°C) and relative humidity (%) was measured at 2 m and 5 cm above soil level using the CS215 probe (Campbell Scientific). The sensor at 5 cm was positioned next to the studied leaves and is corresponding to the leaf level ('plant height'). Soil temperature (°C) was measured at 1 cm and 10 cm soil depth using thermistors (5 kOhm, Epcos, Munich, Germany). In addition, precipitation (mm), volumetric soil water content (Vol%), PAR (µmol m−2 s−1), and wind speed (m s−1) were recorded.

3.3.2 Leaf growth analysis and statistics

The relative growth rate (RGR) was calculated per hour (% h^{-1}) as ratios of pixel area increment (ln ΔA) per unit time interval (Δt, h). Temperature and the other environmental recordings were summarized by calculating means for each hourly RGR time frame. Temperature and relative humidity data at 5 cm (plant height) were used to calculate the vapor pressure deficit (VPD kPa) using the method of Abtew and Melesse (2013).

We modeled RGRs in relation to leaf size (aging) with temperature, vapor pressure deficit and light as response factors using Eq 3.1.

Eq 3.1: $\mathrm{RGR} \sim \sqrt{A} + ((\mathrm{V}.GVx) + (\mathrm{T}.GTx) + (\mathrm{L}.GLx))$,

where A is the leaf area as a nonlinear component to account for leaf aging. The response variables *GVx*, *GTx* and *GLx* (*x* =1-5) are replicate leaf responses to the factors: V (VPD), T (temperature) and L (light); respectively.

The model was developed by constructing step-wise linear models using the above-mentioned variables as predictors. Then, double, triple and all four components were incorporated iteratively. At each step we considered the improvement of the model by ANOVA whereby the final model using all variables as predictors was significantly better than more pauce models. For modeling, we selected a subset of RGR time series above a temperature threshold of ca. 4 °C (5 cm air temperature) in each of the four measurement periods. Above this threshold, our model fitted well the observed RGRs but could not

explain leaf growth below that threshold. Each of the four selected RGR time series had a period of at least 24 hours. For comparison, we used the model parameters to predict relative growth rates and compared it with the observed RGRs. We also analyzed the residuals of our leaf growth model over time and fitted a cosine function to test whether the components that cannot be explained by environmental prediction factors show a circadian pattern.

To compensate for short-term environmental effects on leaf growth, RGRs were calculated also with 24-hour resolution (leaf area increment from 00:00 to 23:59). These daily RGRs were then correlated with the corresponding thermal sums, which were calculated by summing the hourly mean temperatures above 0 °C within the 24 hour periods, divided by 24.

All calculations, statistical analyses and diagrams were performed using R Statistical Software (version 3.0.2; R Core Team 2014) and the packages “ggplot2” (Wickham 2009).

Figure 3.1

Experimental set up of the image based rapeseed leaf growth measurements in the field.

3.4 Results

3.4.1 Environmental field conditions

The studied rapeseed plants were exposed to a fluctuating thermal regime, where temperatures fluctuated between -5 °C and +15 °C causing regular freezing and unfreezing of the studied leaves. The measured temperatures differed in the range of 1 to 5 K depending on the position of the temperature sensor and the time of the day (Fig 3.2 and 3.3). Air temperatures at 2 m height were always higher compared to temperatures at plant height (5 cm, Fig. 3.2 and 3.3). Air temperatures at 5 cm matched with time point of freezing and unfreezing of the studied leaves in the camera images. We therefore considered the temperatures recorded at plant height as more representative for the thermal conditions of the growth measurements and used these temperatures for leaf growth analysis hereafter. Periods with sub-zero air temperatures for more than 24 h were excluded from analyses.

The relative air humidity at 5 cm height was close to saturation during nighttime and decreased to ca. 60% during the day (data not shown). Accordingly, the calculated vapor pressure deficit was close to zero at night and rose during the day to maximum peaks between 0.4 and 0.8 kPa (Fig. 2 and 3).

The soil moisture, measured as volumetric water content was high throughout the study period (mean of 27 ± 2 Vol%, at -10 cm soil depth), which is close to field capacity. In addition, soil temperatures in -1 cm depth (Fig. 2 & 3) and in -10 cm depth (data not shown) were never below 0 °C. Thus, our studied plants were not limited by soil water availability.

3.4.2 Leaf growth

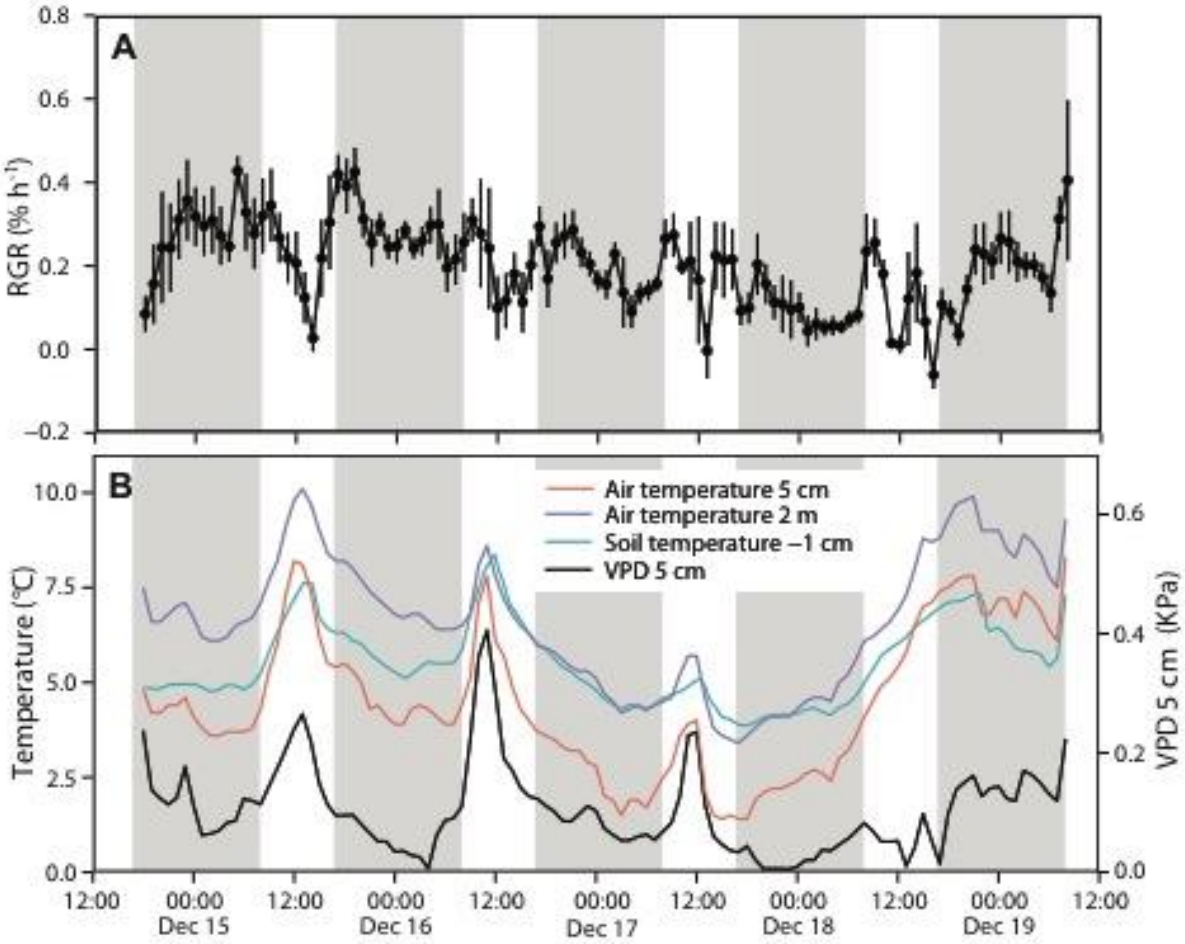
Leaf growth of rapeseed was measured over four weeks in four study periods and is shown as relative leaf growth rates (RGR % h^{-1}). Figure 3.2 shows the period one with temperatures entirely above 0 °C and Figure 3.3 shows the period three with leaf freezing at the beginning and the end. RGRs were positive whenever the air temperature at 5 cm (plant height) was above 0 °C. Leaf growth ceased when temperatures fell below 0 °C (January 11, 00:00 – 12:00, Fig. 3). Freezing and unfreezing of the leaf tissue induced movements of the leaf area and the marker tracking beats which caused artificial positive peaks the RGRs, indicated by arrows in Figure 3.3. These artificial peaks helped to identify

the exact time of freezing and unfreezing. After freezing, the leaves resumed to grow and showed positive RGRs in the next night.

The daily pattern of the RGR showed a pronounced minimum at midday or the early afternoon hours. These RGR minima matched exactly with the daily maximum of vapor pressure deficit and the maximum air temperature at 5 cm (Fig. 3.2 and 3.3). At night, RGRs where mostly higher compared to RGRs at daytime, however, temperatures were similar or even lower during the night (Fig. 3.2 and 3.3). This diel growth pattern was evident when the air temperature (5 cm) did not fall below a value of ca. 4 °C (Fig. 3.2, 3.3 and 3.4). At temperatures below 4 °C, maximum RGRs were between 0.2 and 0.3 mm h^{-1} independent of daytime and no such diel pattern was observed.

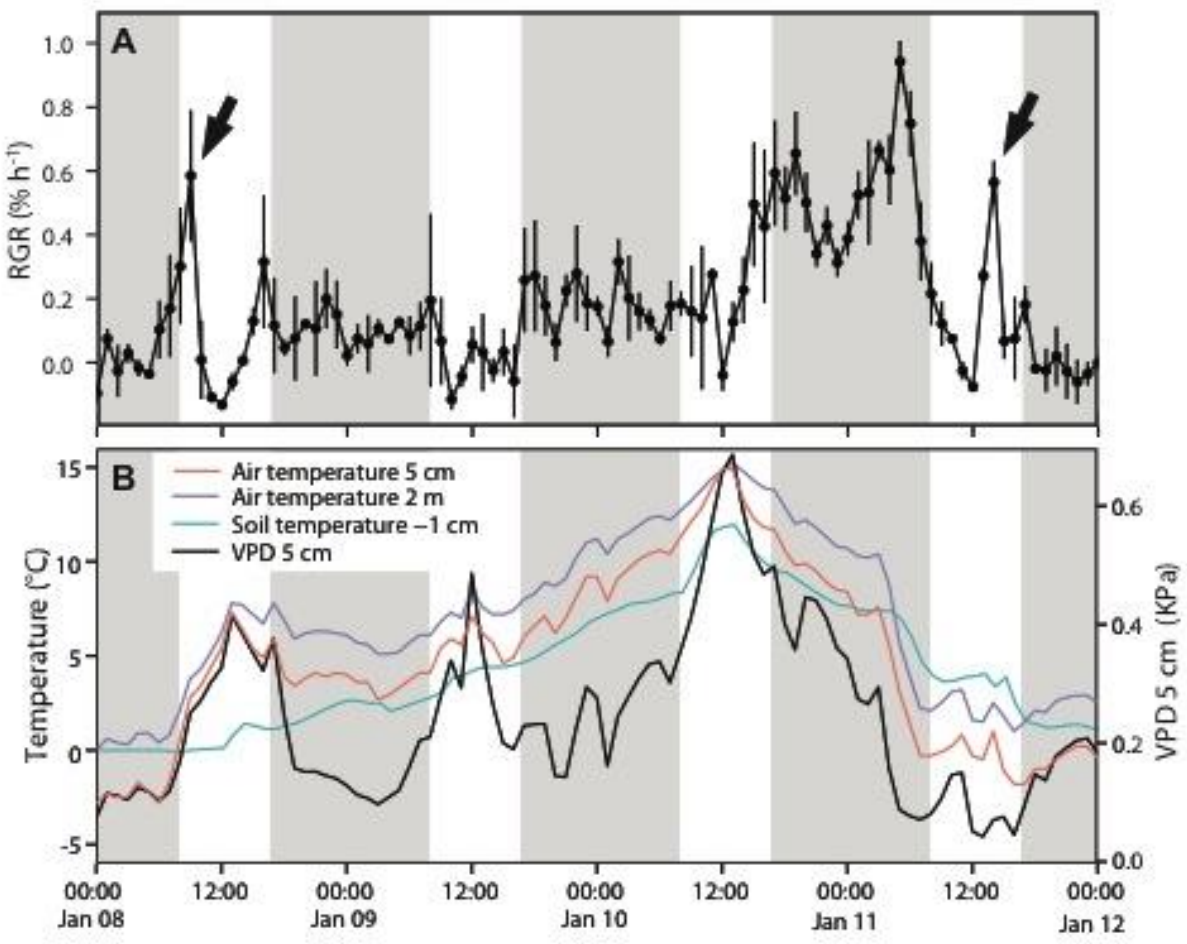
For leaf growth modeling, we selected the periods with temperatures above 4 °C where RGRs showed the above mentioned diel growth pattern. The RGRs predicted by modeling matched the observed RGRs with an R^2 of at least 0.31 ($P<0.001$, Fig. 3.4). During the day, the predicted RGR followed the measured RGRs precisely. At night, the model derived RGR fitted the measured RGR less well. The RGR particularly in the early dark period could not be predicted by our model based on environmental conditions (Fig. 3.4). Across all four study periods, VPD had always the most significant negative effect on leaf growth whereas air temperature at 5 cm had only a significant positive effect in the measurement period 2 and 3. Solar radiation was significantly negatively correlated with leaf growth, presumably because of its co-variation with VPD, and thus turgor. As a consequence RGR was higher during the night (Tab. 3.1). Leaf area as a model factor was significantly negatively correlated with RGRs in period 1 and 2 and showed a negative trend in period 3 and 4. Thus, leaves grew slower the bigger, respectively the older they got. The residuals of our leaf growth model showed a rhythmic oscillating pattern over time ($R^2 = 0.24$, $P < 0.001$, Fig. 3.5). The mean RGRs calculated over 24 h of all four measured periods were significantly correlated (linearly) with the sum of °h above 0 °C. The x-axis intercept of the regression line was close to 0 °C, which we consider as the base temperature for winter rapeseed (Fig. 3.6).

Figure 3.2



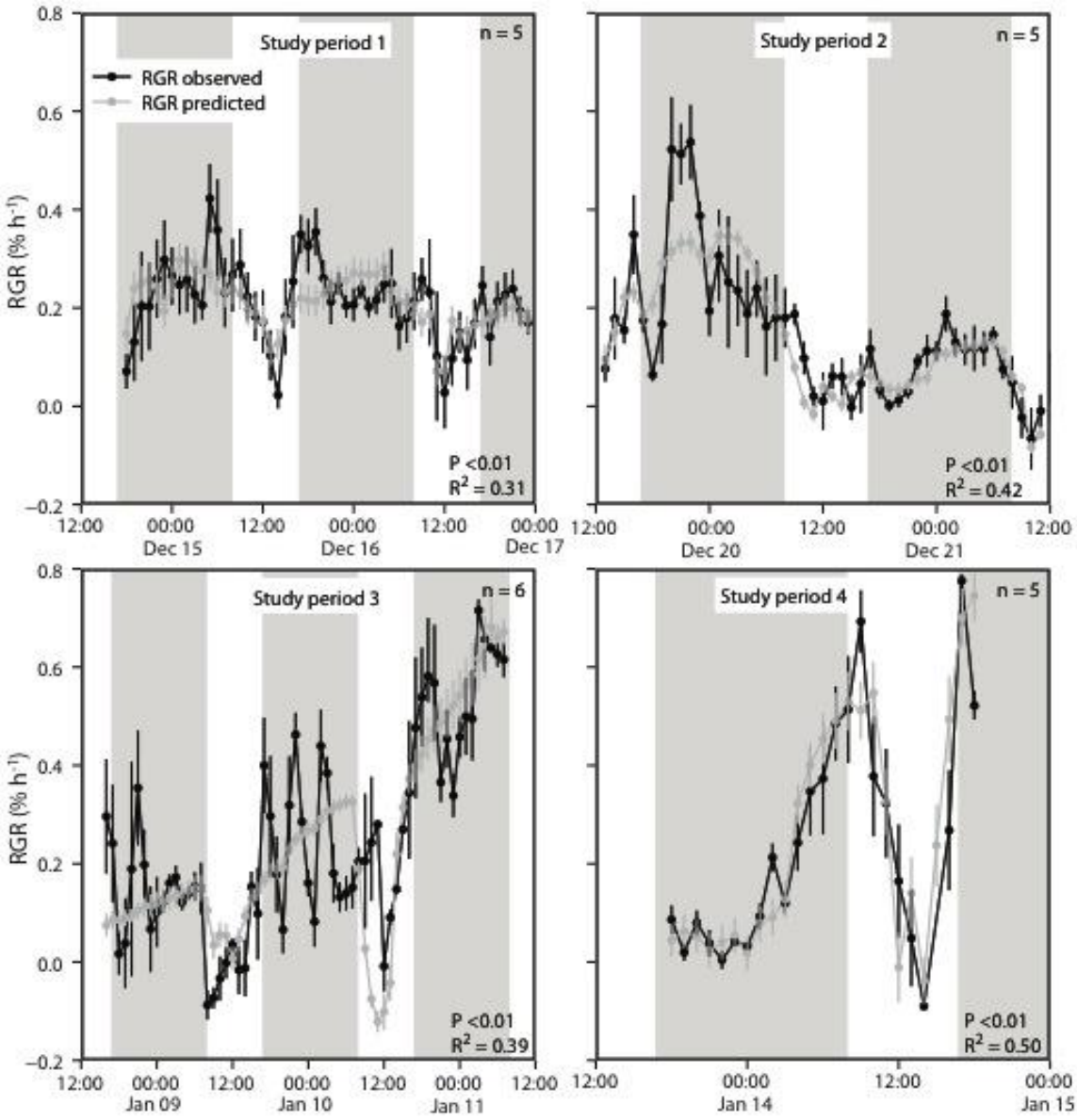
Time series of rapeseed leaves measured for four and a half days with temperatures above 0 °C (study period 1**). A** shows the mean relative growth rate (RGR % h^{-1}, n = 5 leaves) with bars showing the standard deviation between leaves. B shows temperatures measured at three different positions (left Y-Axis) and the calculated vapor pressure deficit (VPD) at plant height (right Y-Axis).

Figure 3.3



A four day time series of growing rapeseed leaves (study period 3) with leaf freezing at the beginning and end, indicated by black arrows **A** shows the mean relative growth rate (RGR % h^{-1}, n = 5 leaves) with bars showing the standard deviation between leaves. B shows temperatures measured at three different positions (left Y-Axis) and the calculated vapor pressure deficit (VPD) at plant height (right Y-Axis).

Figure 3.4

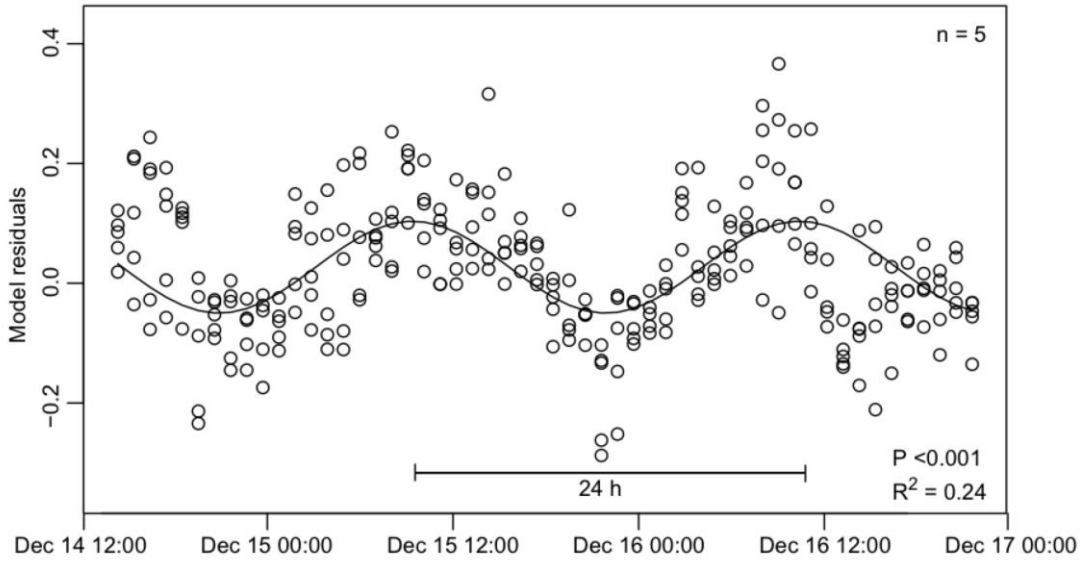


Time series of the relative growth rate (RGR % h^{-1}) for periods during which air temperature at 5 cm never fell below 4 °C in each of the four study periods (black color). P and R^2 values from the leaf growth model are given, from which the predicted RGR (gray color) was derived. Note the significant drop of the observed RGR during the day, which is well described by the predicted RGR.

Table 3.1: Model statistics of RGR (% h^{-1}) in response to the factors: Leaf Area (age), VPD Temperature and solar radiation of the four selected growth time periods above 4.5 °C in each measurement period 1-4.

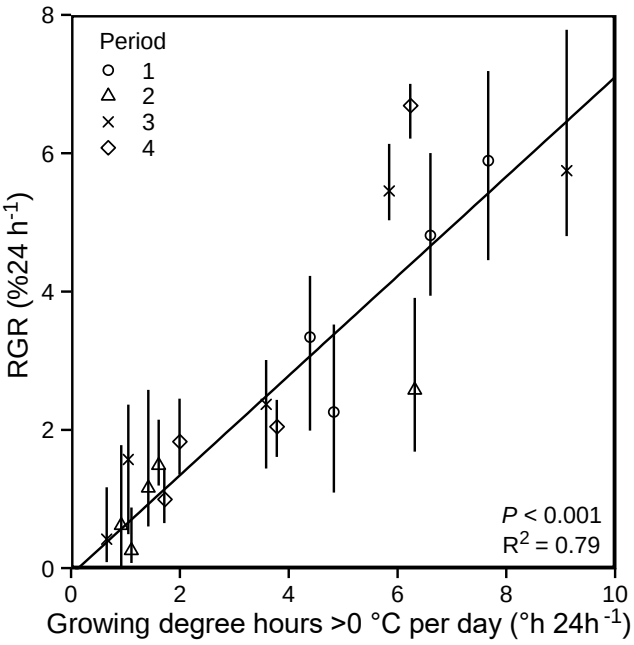
Measurement period	Factor	Estimate	*P*-Value
Period 1, n = 5 leaves	Intercept	2.24	<0.001 ***
	Leaf area	-0.01	<0.001 ***
	VPD	-0.87	<0.001 ***
	Temperature	-0.01	0.27
R^2 = 0.31	Solar radiation	-0.02	0.001 **
Period 2, n = 5 leaves	Intercept	0.27	<0.001 ***
	Leaf area	-0.01	0.03 *
	VPD	-0.71	<0.001 ***
	Temperature	0.03	<0.001 ***
R^2 = 0.42	Solar radiation	-0.01	<0.001 ***
Period 3, n = 6 leaves	Intercept	0.36	0.001 **
	Leaf area	-0.01	0.18
	VPD	-0.68	<0.001 ***
	Temperature	0.04	<0.001 ***
R^2 = 0.39	Solar radiation	-0.01	0.18
Period 4, n = 6 leaves	Intercept	0.90	<0.001 ***
	Leaf area	-0.01	0.13
	VPD	-1.49	<0.001 ***
	Temperature	0.01	0.82
R^2 = 0.50	Solar radiation	-0.01	<0.001 ***

Figure 3.5



Time course of model residuals regressed by a cosine function with 24 hours wave length shown for study period 1. Number of replicate leaves = 5.

Figure 3.6



Daily mean relative leaf growth rates (RGR % 24^{-1}) correlated with the growing degree hours above 0 °C per day divided by 24. Different symbols indicate data points from the four measurement periods. Bars show the standard deviation of the replicates per day in each period. Number of replicate leaves: Period 1, 2 and 4 n = 5; period 3, n = 6. Periods with subzero temperatures for longer than 24 hours were excluded from the analysis.

3.5 Discussion

In this study we monitored leaf growth of winter rapeseed in a naturally fluctuating field environment. The image sequence analysis approach "Martrack Leaf" (Mielewczik et al. 2013) using artificial maker trackers (white beads) attached to the leaf margins allowed to record highly resolved RGRs even under harsh winter conditions. Such growth measurements at critically low temperatures are important to understand thermal growth limitations in dicot winter crops and were not achieved under field conditions so far.

Temperature is a crucial factor to discuss plant growth/temperature relations. The measured air temperatures at 2 m height (standard in meteorology) were always higher compared to the air temperatures at plant height (5 cm), which we use for analysis. This underlines the well-known fact that modeling leaf growth from environmental variables has to take realistic temperatures at or close to the growing leaves into account in order to avoid misinterpretations.

The leaf growth data are discussed first for temperatures between 0 and 4 °C and thereafter for temperatures above 4 °C where we found a clear diel growth pattern which we could well describe by developing a model based on environmental factors.

Below 4 °C, RGRs were small but still positive which was not resolved so far by using daily ruler measurements (Körner 2008). The leaf growth rates at hourly and daily resolution indicate an absolute base temperature for winter rapeseed close to 0 °C (Fig. 3.3 and 3.6), which is similar to the thermal growth limit of graminoid winter cereals (0 to 1 °C, Gallagher 1979, Gallagher and Biscoe 1979, Nagelmüller et al. 2016). Also for winter rapeseed leaf growth, earlier studies with less temporal resolution concluded on base temperatures between 0.4 and 1.2 °C (Vigil et al. 1997, Hodgson 1978).

The studied leaves here recovered rapidly from freezing within a few hours. However, we cannot differentiate, whether the low growth rates at daytime hours following such freezing events were due to freezing recovery processes or due to VPD-induced effects that would have resulted in growth minima at that time of the day also in case of non-freezing prior conditions (Fig. 3.3).

When temperatures were constantly above ca. 4 °C, a diel growth pattern with maximum RGR at night was detected. This pattern is a typical leaf growth pattern reported for leaves of several dicot species at higher temperatures (Walter et al. 2009, Poire et al. 2010). Here, it occurred even though temperature was lower during the night compared to the day. This

result was surprising because we expected that temperatures close to the thermal limit would have a stronger restricting effect on leaf growth.

At peak temperature, VPD negatively affects leaf growth (Tab. 3.1), which explains the depressions of the RGRs during daytime, leading to the observed diel growth pattern (Fig. 3.4). These RGR minima during the day suggest that VPD peaks enforce the evaporative demand of plants during daytime even at the low temperature conditions of our experiment in a similar way as in studies performed at higher temperatures (Sadok et al 2007, Ben-Haj-Salah and Tardieu 1996). Thus, VPD may counteract the positive effect of temperature on leaf growth even at low temperature. However, the calculated VPD peaks under winter field conditions are well below VPD values that were found to restrict leaf growth at optimal temperatures (Durand et al. 1995, Sadok et al. 2007). VPD might also have a stronger negative effect on leaf growth in dicot species compared to monocots at low temperature conditions. Winter cereals grown at comparable thermal field conditions showed no such growth depression during the day and leaf elongation rates are highly positively correlated with temperature (Gallagher and Biscoe 1979, Gallagher et al. 1979, Nagelmueller et al. 2016). The process of cell expansion in dicot species might be less temperature sensitive and is independent from cell division- or cell wall formation processes (Tardieu et al. 1999, Parent and Tardieu 2012). Thus, cell expansion can be shifted to time points characterized by colder conditions as e.g. during the night, when evapotranspiration does not restrict turgor-driven expansion of vacuoles, cells and leaves. Such growth peaks, which we observed during the night, are underestimated by our model (Fig. 4) and might be explained by endogenous factors such as regulatory mechanisms connected to the circadian clock (Ruts et al. 2012).

Evidence for such a diel or circadian component is indicated by the regularly oscillating residuals over time of our environmental data based growth model (Fig. 3.5). The circadian clock plays an important role in regulating the diel pattern of leaf growth (Webb 2003, Farré 2012, Ruts et al. 2012) and most of the genes involved in growth processes are circadian expressed (Covington 2008). Low temperature is known to disrupt circadian cycles, to lower the amplitude of the circadian clock contribution and to change the expression of clock related genes, as it was found in *Arabidopsis* (Bieniawska et al. 2008). Thus, temperatures below 4 °C, at which the rapeseed leaves show no diel leaf growth pattern, could represent a physiological barrier for maintaining circadian controlled processes. However, the precise modeling of the influence of such endogenous factors at

low temperature field conditions is not possible based on our data and the underlying molecular basis is awaiting clarification in future studies.
Integrating leaf growth over 24 hours, compensated for the hourly fluctuation of RGRs caused by the changing environmental conditions or putative clock regulations. Such daily growth increments are significantly positively correlated with the thermal sums above the base temperature of 0 °C, confirming a wide body of literature relating leaf growth and plant development to temperature as measured in daily or longer increments (thermal time concept; see e.g. studies by Milford et al. 1985, Körner 2008).
Our data show that rapeseed, as a typical dicot winter crop does grow below temperatures of 5 °C which is used as a threshold for common growing season definitions (Frich et al. 2002, Carter 1998). We can even specify that rapeseed grows with a typical dicot diel growth rhythm above 4 °C and shows smaller growth rates when temperatures approach 0 °C. However, those small leaf area increments should not be neglected since they can accumulate to significant amounts of biomass when considering longer time periods such as an entire winter season. Studying leaf growth dynamics in relation to low temperature winter conditions is and will become more relevant in the context of climate change. Growing seasons have increased in length and periods with warmer temperatures during winter dormancy have become more frequent (Menzel and Fabian 1999, Frich et al. 2002, Robeson 2002). Even a small positive change in temperature and thus, increasing thermal time promotes the production of biomass and yield in rapeseed and other winter crops (Miralles et al. 2001). Well-adopted cold tolerant varieties might hold a higher yield potential by increased biomass accumulation during winter and a better competition against weed through higher canopy cover, which could be exploited by breeders.

3.6 Acknowledgements

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4 Low temperature limits for root growth in alpine species are set by cell differentiation

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Cell elongation, functional growth analysis, lignification, low temperature, root growth, sink–source relationships, xylem.

4.1 Abstract

Plant growth in cold climates is not limited by carbon assimilation (source activity) but rather by reduced carbon investment into new tissues (sink limitation). It has been hypothesized that all cold adapted plants face similar growth constrains at low temperature mainly associated with the formation of new tissues. To explore the thermal limitation of plant tissue formation, we studied root growth and anatomical root tissue characteristics in four cold adapted alpine species, grown in thermostated soils with a vertical temperature gradient approaching 1°C. Above-ground plant organs were exposed to typical alpine climate conditions (high solar radiation and cool nights) at 2440m a.s.l. in the Swiss Alps to assure continuous source activity. Image based measurements of root growth (root elongation rates at 12-h-intervalls, RERs) were combined with anatomical examinations in thermally constrained root tips as well as with a functional growth analysis of entire plants. Temperatures in the range 0.8 to1.4°C were denoted as critically low temperature thresholds for root formation across the four species. The RERs $12h^{-1}$ revealed that roots kept extending at very low rates just above this threshold but cell elongation and xylem lignification were clearly inhibited in the terminal zones of root tips. Because of the very reduced elongation rates between 1 and 5°C, roots produced in that range of temperatures contributed very little to the entire root-system compared to control roots grown at 10°C. Hardly any secondary roots were formed at temperatures below 5°C and total root mass was substantially lower (74% reduction in comparison to control) as well as above-ground biomass was reduced by 23%. Cell elongation and differentiation rather than cell division control root length increment and the size of root cells at the low temperature limit of growth. Lignification of root xylem is clearly constrained at temperatures below 3°C.

4.2 Introduction

In arctic-alpine environments, low temperatures constrain the growing season and thus biomass production of plants (Biss 1956, Pollock and Eagles 1987, Körner, 2003). Winter crops and plant species in grasslands revealed very similar growth limitations to cold temperatures (Gallagher 1979, Wingler and Hennessy 2016), therefore, it has been hypothesized that all cold adapted plants underlie common growth constrains when temperature arrives at a critical threshold (Körner 2008). Above zero soil temperatures need to occur over at least 6 weeks for angiosperm survival (Körner 2011). Results of earlier works have indicated that tissue formation, irrespective of whether above or below-ground, becomes very slow at or below 5 °C (Alvarez-Uria and Körner 2007, Körner 2008, Nagelmüller et al. 2016a) and was never observed at or below 0 °C, a temperature that still permits CO_2 uptake at ca. 30 % of photosynthetic capacity. Hence, at such low temperatures plant growth is not carbon limited (Pollock et al. 1988, Xiong et al. 1999, Körner 2003, 2015). Similar low temperature thresholds were reported for leaf expansion as well as for root length increment (Körner and Woodward 1987, Schenker et al. 2014, Nagelmüller et al. 2016a), and radial growth of xylem (Rossi et al. 2007), suggesting that apical and lateral meristems exhibit similar temperature responses and face the same low temperature limitations at tissue and cell level. Leaves of cold adapted Poaceae start expanding very slowly at close to 0 °C (Peacock 1975, Körner and Woodward 1987, Porter and Gawith 1999, Nagelmüller et al. 2016b). Although, absolute minimum temperature thresholds for growth do not explain the overall plant performance in cold climates, the analysis of tissue processes at such extreme thermal constraints provides insights into the underlying physiological and anatomical mechanisms that control life at the cold edge.

The production of new plant tissue includes cell division, cell enlargement and cell differentiation into various operational cell types (in that sequence). From what is known to date, cell division in cold adapted plants is not interrupted at close to 0 °C (Francis and Barlow 1987, Körner and Pelaez-Menendez-Riedl 1989). Cell enlargement depends on balanced rates of turgor driven cell wall expansion and secondary cell wall synthesis. In graminoids, water flux into the vacuole (a major driver of cell expansion) does not appear to be affected over a temperature range from 2 to 20 °C (Thomas et al., 1989, Pollock et al. 1990). Even in a chilling sensitive cucumber, vacuoles exposed to 8 °C had no problem to absorb water (Lee et al. 2005), and *Spinacia* plants rapidly adopted root hydraulic pressure after root temperature was reduced from 20 to 5 °C (Fennell and Markhart 1998). Hence,

the critical processes are mainly to be associated with the growing cell wall. In the expansion zone of shoots and roots, cells undergo a several-fold size enlargement, which cannot be achieved with the initial primary wall. Secondary wall formation must go hand in hand with size increment, so, cell enlargement cannot be separated from differentiation, the most resource demanding process (Pollock and Eagle 1987). As part of that differentiation xylem and phloem become established. Xylogenesis notably contributes to the final biomass because of the thick xylem cell walls and their lignification. A low temperature driven slowing of cell differentiation must feedback on cell division in order to retain mechanical robustness of the resulting tissue (Körner 2003). In conifers near treeline, xylogenesis was found to cease at temperatures below 4 – 5 °C (Rossi et al. 2007, 2008). A lower temperature threshold for xylogenesis (2.0 ± 0.6 °C) was recently reported in the alpine shrub *Rhododendron aganniphum* (Li et al. 2016). Yet, we are dealing with an asymptotic decline, causing the absolute limit to become a matter of precision and definition. We suspect that cell differentiation (including lignification) is the most likely cause of root growth cessation at very low temperatures which otherwise still enable photosynthesis and cell division.

To explore these processes at tissue and cell level, we decided to use roots and root tips because roots grow in a thermally buffered environment, permitting to explore the effect of even minute temperature differences on meristematic activity at critically low (still positive) temperatures. Roots expanding from ambient soil surface temperatures towards critically cold conditions deeper in the soil allow identifying threshold temperatures and also to sample root tips developed under such cold conditions (Alvarez-Uria and Körner 2007, Schenker et al. 2014). We exposed four alpine plant species to such conditions in the field. From a prior research, employing cold glacier water runoffs as cooling medium we delineated that the zero point for root growth is below 5 °C, however, a precise minimum temperature threshold could not been defined nor could the tissue level responses be assessed for the thermal limit of growth (Nagelmüller et al. 2016a). In the present study, we quantified anatomical/histological changes of cell expansion/differentiation in roots and root tips (root kinematics, Silk and Erickson 1979, Sharp et al. 2004) grown at precisely controlled temperatures below 3 °C in order to identify the absolute minimum temperature threshold for root growth and cell elongation and differentiation. We expected a continuous cell division but a delay in the rate of cell enlargement and cell differentiation, causing this zone of the root tip to lengthen relative to

controls at 10 °C. We also anticipated a weaker lignification, hence, a longer stretch of poorly lignified tissue behind the root tip as it reaches its low temperature growth limit.

4.3 Material and Methods

4.3.1 Experimental setup

The experiment was conducted at the ALPFOR research station, close to the Furka Pass, at 2440 m a.s.l. in the Swiss central Alps. Individuals of four alpine plant species, *Ranunculus glacialis* L. (Ranunculaceae), *Rumex alpinus* L. (Polygonaceae), *Tussilago farfara* L. (Asteraceae) and the grass species *Poa alpina* ssp. *vivipara*, L. (Poaceae) were collected at a very early seasonal developmental stage. We selected plantlets with newly emerging root tips of less than 2 mm length on the day of sampling. Roots from the previous growing season were cut to 3 cm length for later distinction from newly developed roots. For each species, we planted 42 individuals in cylindrical containers so that the apical meristem was positioned at -1 cm soil depth, which also correspond to the position of the youngest newly emerging root tips.

Half of the plants were planted in Plexiglas® cylinders (200 x 50 mm, 1 mm wall thickness, Evonik Industries, Essen, Germany), which allowed image based root elongation measurements. The other half was planted in correspondingly sized polypropylene tubes with 0.8 mm wall thickness (‘p-Safe PP’, 5-P KG, Sulz, Germany), appropriate for measuring final rooting depth and the final harvest of root tips as well as the total root biomass. Both types of cylinders had a watertight seal at the bottom. The lowest 2 cm of each cylinder were filled with quartz sand (grain size: 2 mm) for drainage water, separated from the growth substrate by a fibre mat (Fig. 4.1). The upper 18 cm of the tube length was filled with a substrate mixture of 80 % fine sandy glacier silt and 20 % potting compost (Capito Universalerde, Fenaco, Bern, Switzerland). Fertilizer was provided weekly (in total three times over the 29-day-experimental period) by adding 10 ml full strength Hoagland's solution (1.6 g L^{-1} of Hoagland salts; Sigma-Aldrich, Munich, Germany). Plants were watered with 20 ml water every second day in case there was no precipitation. Excess water (also from rain) drained to the bottom, was removed with a hand pump, using a 3 mm tube that reached the cylinder bottom.

Plants were exposed for 29 days during the main part of the growing season to have low and high substrate temperatures by immersing the cylinders into four double-walled 96 L stainless steel, thermostated water tanks (interior dimensions: 80 × 60 × 20 cm). Three of these water baths were set to 1 °C and one to10 °C as a control, resulting in temperatures of ca. 1.5 and 10.3 °C of the circulating cooling water (see result section). Thirty plant replicates per species were placed in each of the low temperature baths, and 12 plant

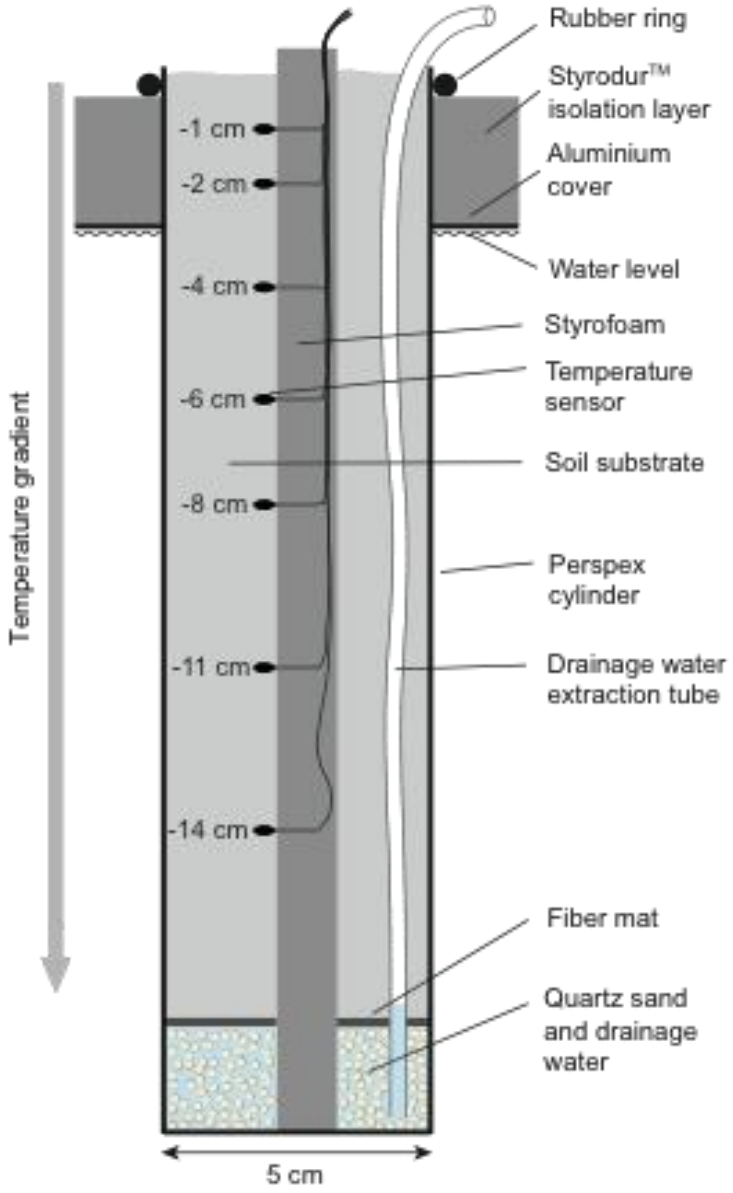
replicates per species were placed in the 'warm' control bath. Cylinders were randomly arranged in the water baths.

Each water bath was equipped with a thermostat system (CBN 28-30 and HTM 200, Heto-Holten, Allerød, Denmark) and a water-circulating system to ensure uniform temperature distribution in the water baths. To minimize vertical heat flow, water baths were covered by a 2 mm aluminium plate and with a 2 cm Styrodur^{TM} isolation layer on top. These covers had 50 mm diameter holes into which 40 cylinders per bath were inserted and fastened by a rubber ring (Fig. 4.1). The rings also prevented light from leaking into the "below-ground" compartment. The water bath systems were placed on the terrace of the ALPFOR station to expose above-ground plant organs to typical alpine climate conditions (Fig. 4.2; [Supplementary Information, Fig. S4.2]).

The soil temperature gradient was measured in two cylinders per water bath (named T-cylinders each equipped with seven small temperature sensors (NTC-resistors, 2 mm in diameter, 5 kOhm at 25 °C, ±0.2 K, Epcos, Munich, Germany) at depths from -10 to -140 mm in the cylinders (Fig. 4.1) and with 1 cm distance to the cylinder wall. Plants were absent in these T-cylinders. Water temperatures in each water bath were measured by another set of NTC-resistors close to the water-circulating system. Temperatures in T-cylinders were recorded every 10 minutes with a data logger (CR1000, Campbell Scientific, Logan, UT, USA) and two AM16/32B multiplexers (Campbell Scientific) and hourly temperature means were further used for all calculations.

Due to short periods of equipment failure early in the experiment, the water in the cold treatment bath 3 heated up to 19 °C for 14 hours on treatment-day-3 and up to 9 °C for seven hours on treatment-day-9 [Supplementary Information, Fig. S4.1]. In the warm control treatment, water heated up to 21 °C for 12 hours on treatment-day-1. The brief temperature deviations emerged before RER measurements started, and except for the seven hours on day-9, occurred immediately after planting, when plants were still affected by the transplantation.

Figure 4.1



Design of a plant cylinder with temperature sensors placed at different soil depth.

Figure 4.2

Thermostated water bath systems at the Furka Pass (2440 m a.s.l.) with transparent cylinders for root observation (measurements of RERs). The black to white converted image of a root cylinder taken in a photo-box shows roots of *Rumex alpinus* growing along the wall. White arrows point at root tip positions.

4.3.2 Growth measurements

Root elongation rates (RERs) were obtained for a period of nine days and started on treatment-day-14 when sufficient roots arrived at the transparent cylinder walls of the cold treatment. Digital images (25 pixel mm^{-1}) of the Plexiglas® cylinders were taken in 12 h intervals (07:00 in the morning and 19:00 CET in the evening) by using a photo box (100 x 60 x 60 cm) equipped with a digital camera (Nikon D7000, Nikon, Tokyo, Japan) with a 35 mm lens (Nikon) and a flash (64AF1, Metz, Zirndorf, Germany) for illumination. To ensure consistent image frames, cylinders were placed at exactly the same distance and orientation using positioning guides. Collecting and repositioning cylinders and taking photos took less than 90 s.

The position of root tips was tracked across the sequence of images by using the software "ImageJ" (version 1.47v, Rasband 1997-2015) and the plug-in "SmartRoot" (Lobet et al. 2011). We measured root length increment to calculate RERs (mm 12 h^{-1}) and the root tip position in the cylinders (soil depth). The root tip positions were related to the temperature profiles along the cylinders [Supplementary Information, Fig. S4.3].

All plant individuals were harvested after 29 treatment days. Both types of cylinders were opened at the bottom. The substrate was carefully removed until the tip of the single, deepest root became visible (without stretching the root) and maximum rooting depth was measured with a ruler. These depth values (averaged from several single deepest roots of cylinders) were used to calculate the mean minimum temperature threshold for root growth for each species. Thereafter, plants were washed and photographed and separated into roots, leaves and stems including flowers. Leaves and roots were scanned with a transmitting light scanner (Epson Expression 1680, Epson, Meerbusch, Germany). Total root length, numbers of primary and lateral roots were calculated from scans, using the WinRHIZO software (Regent Instruments INC., Quebec, Canada). Dry weight was obtained after drying at 80 °C for at least 48 h. Specific root length (SRL) and specific leaf area (SLA) were calculated by dividing the total root length (m) and leaf area (cm^2) by the corresponding dry weights (g). For biomass allocation (functional growth analysis), mass fractions (leaf, stem and root mass fractions) were calculated by dividing the dry weights of the fractions by the total plant weight.

4.3.3 Root anatomy

Longitudinal thin sections (80 µm) of the 2 cm front of the root tips were made through the central cylinder of roots to assess (i) cell density per unit root area, (ii) cell length in the elongation zone, and (iii) cell differentiation (lignification) as described in the following. We sampled root tips from several single deepest roots of different cylinder at the day of harvest and stored them in 75% (v/v) ethanol. Root tips were cut in pieces of five mm length, embedded in 3% (w/v) agarose gel and cut by a vibratome (VT1200, Leica Biosystems, Nussloch, Germany). To visualize lignification in the xylem, thin sections of 80 µm were stained following Brundrett et al. (1988) starting with 1 h in 0.1% (w/v) berberine hemi-sulphate followed by 30 min in 0.5% (w/v) aniline blue at room temperature. Stained sections were mounted on microscope slides in 50% (v/v) glycerine with 0.1% (w/v) of $FeCl_3$ as a preservative. Sections were viewed with a fluorescence microscope (Leica DM 2500, Leica Microsystems, Wetzlar, Germany) equipped with an UV-filter set (excitation filter BP 320-280 nm, chromatic beam splitter FT 400 (400 nm), emission filter LP 425 nm). For image analysis, series of overlapping images were taken along the root by a digital microscope camera (Leica DFC 300 FX, 3.2 pixels mm^{-1}) with constant 100 ms exposure time. Single images were merged by eye with the program "Illustrator CS5" (Adobe Systems Incorporated, San Jose, CA, USA) to display a longitudinal section of the entire 20 mm root tip.

We (i) counted the number of cells in a 0.2 x 0.2 mm square which was positioned as close as possible to the root meristem's initials (root apex, beneath the root cap), determined (ii) the mean final cell length in roots, measured (iii) the distance from the root apex to the position at which final cell length was reached to define the length of the cell elongation zone, and (iv) assessed the degree of lignification of the xylem along the root. For that (iv), we have selected four image snippets (0.6 mm in diameter) of the central cylinder every 4 mm along the root starting at the root apex. Pixels of image sections were examined for lignification over a defined strip of 10 pixel width (ca. 0.2 mm) over the length of the four snippets using MATLAB 8.2 (The Mathworks, Natick, MA, USA). Then, we extracted the L-channel values of the HSL colour space (H= hue, S= saturation and L = index of lightness, with values between 0 and 1, RGB red-green-blue colour model) of the selected pixels and averaged these values by the number of pixels. These light intensity indices were used as a quantitative proxy for lignification. In addition, we measured (5) the distance from the root apex to the first lignified (fluorescent) xylem

element. Cell size and distance measurements were done using the program “ImageJ” (see above).

3.3.4 Data analysis and statistics

We calculated the correlations between soil temperature and soil depth for each water bath and the measured RER during 12 h (mm 12 h^{-1}) as well as the root tip positions at harvest using third order polynomial regressions as the temperature decrease between -20 and -80 mm soil depth was nonlinear. Root tip temperatures were then estimated using the polynomial functions, based on the root tip positions at the end of each 12 h interval. Hourly temperatures within each RER 12 h interval were used for the polynomial fit as well as hourly minimum and maximum temperatures during the 12h interval were considered [Supplementary Information, Fig. S4.3]. Since root tips occurred over a narrow range of the profile only (with curve fitting outside that range not relevant for root tips), we also estimated root tip temperatures derived from two combined linear regressions between the sensor depths -20 to -40mm and -40 to -60 mm, where most cold treated roots grew. These temperatures differed from temperatures from the polynomial regressions on average by ± 0.017 K (sd) with a maximum deviation of 0.02 K. Since this deviation is below the T-sensor accuracy, we are confident that the polynomial regressions reflect the root tip temperature in the root observation window with the needed precision. To analyse the relationship between RERs (mm $12h^{-1}$) and the root tip temperatures, linear models were applied. The minimum temperature for root growth was derived from the single deepest root within a cylinder reached at the day of harvest (after 29 full treatment days). Here, we used for the polynomial fit of all hourly temperatures after the last RER measurement until the root harvest. We also calculated a mean by averaging the deepest root tip positions of several cylinders per species and the corresponding temperatures at these positions.

To test for differences between temperature treatments, we performed one-way ANOVAs for: RER, total root length, root dry weight, SRL, number of primary and secondary roots, below-ground biomass (BGB), leaf area, leaf mass, SLA, above-ground biomass (AGB), biomass fractions, the lengths of the root elongation zone and the distance from root tip where first lignified xylem was detected. Additionally, we performed a linear model to test for differences in light intensity of the fluorescent xylem between temperature treatments plus for the light intensity increase along the root length (nested design) for each species. For the analysis of the post-harvest data, we merged the data from

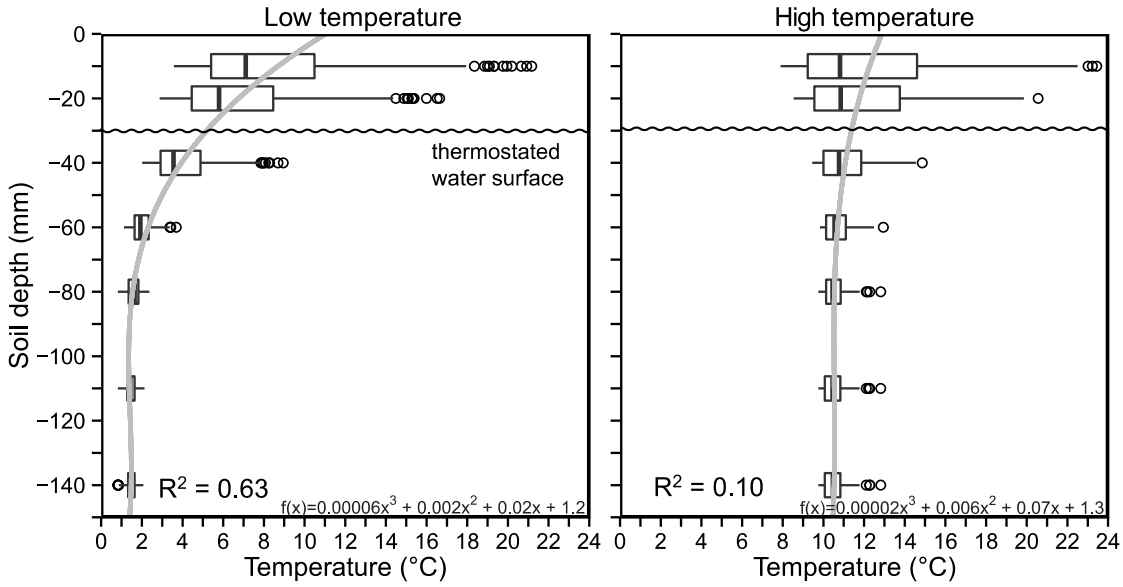
the three cold water baths since no significant differences we found among water baths (n.s. for factor bath). Number of replicates for different traits often deviated from the number of replicates at the beginning of the experiment, since individuals varied in the performance of certain traits. The normal distribution of post-harvest data was tested visually (q-q plots, histograms) and as log-transformation did not yield different statistical outcomes, non-transformed data are here presented. All statistical analyses and diagrams were done with R Statistical Software (version 3.0.2; R Development Core Team, 2014) and the package “ggplot2” (Wickham 2009).

4.4 Results

4.4.1 Soil temperature

The cooling water bath systems provided a hourly mean of water temperatures of 1.5 ± 0.4 °C (± sd) in bath 1, 1.5 ± 0.6 °C in bath 2, 1.4 ± 0.9 in bath 3 during the experiment, underpinning that the temperature regime among the three baths was identical. The mean temperature for all hourly intervals of the warm water bath was 10.6 ± 0.5 °C. The temperature in the cylinders declined with soil depth in both treatments. In the cold treatment, this temperature decrease was particularly pronounced between -10 and -80 mm (R^2 =0.96, P <0.001, Fig. 4.3) and most cold treated roots grew not deeper than -80 mm. Temperature decreased from a hourly mean of 8.5 ± 4.3 °C at the top (-10 mm, ± sd) to 1.5 ± 0.8 °C at -80 mm depth and the temperature further approached 1 °C below -80 mm soil depth. In the warm treatment, the temperature gradient was less steep, ranging from a hourly mean of 12.2 ± 3.9 °C at -10 mm to 10.6 ± 0.5 °C at -140 mm (R^2 = 0.72 ± 0.23, P <0.001, Fig. 4.3). The diurnal temperature fluctuations in the upper 40 mm of the soil column were caused by fluctuating solar radiation in both, cold and warm treatments, especially, since the uppermost 30 mm of the cylinders were not immersed in the water but insulated by the Styrodur layer. At -60 mm soil depth, these fluctuations became minor and the temperature differences of 8-10 K between the cold and warm treatment were stable (Fig. 4.3, [Supplementary Information, Fig. S4.1]). In the deepest layers where roots still grew, the hourly maximum temperatures never surpassed 5.2 °C at -60 mm, 2.2 °C at -80 mm and -110 mm soil depth in each of the three cold water baths.

Figure 4.3



Polynomial temperature profiles (grey line) in cold (left) and warm (right) treated plant cylinders calculated during measurements of root elongation rates (RERs). Data of the three cold water baths were averaged. Boxplots display the hourly temperature readings for each sensor depth. Boxes comprise the 25–75 % quantiles with the median as vertical line, dots indicate outliers. The deepest temperature sensor was located at -140 mm depth. Similar polynomial regressions were made for each RER $12h^{-1}$ to estimate root tip temperatures.

Table 4.1: Root length responses near the low temperature limit. Root elongation rates (RER) during the 12 h interval with the lowest temperature in the cold treatment, the deepest single root tip position and the mean maximum root tip depths (averaged across cylinders per species ± sd) at harvest. Corresponding root tip temperatures were derived from the temperature gradients (polynom functions, calculated for 12 h for RER and for the last five treatment days prior to harvest)

	Rumex	*Ranunculus*	*Tussilago*	*Poa*
RER (mm $12h^{-1}$)	0.4	0.2	0.3	0.6
Temperature (°C) during 12h-interval	0.9 (0.8-1.1)	0.9 (0.7-1.2)	0.7 (0.6-1.1)	0.7 (0.7-1.0)
Single deepest root tip position (mm)	73.7	77.3	87.6	105.8
Root tip temperature (°C; hourly min - max)	1.2 (1.1-1.4)	1.0 (0.8-1.2)	1.0 (0.9-1.3)	1.0 (0.8-1.3)
Mean of maximum root tip positions (mm; ± sd)	54.4 ± 11.9	61.5 ± 12.3	65.5 ± 14.5	91.7 ± 13.1
Number of deepest root tips (n)	n=26	n=30	n=26	n=29
Mean of root tip temperatures (°C; ± sd):	2.4 ± 1.1	2.0 ± 0.9	1.9 ± 0.8	1.5 ± 0.3
Hourly min / max temperatures#	1.1 / 5.3	0.8 / 5.1	0.9 / 4.7	0.8 / 2.8

Hourly minimum and maximum temperatures for n=26-30 single roots per species during root formation

3.4.2 Root length increment and temperature

Root elongation rates (RERs) per 12 h interval in the cold treatment were linearly and positively correlated with the mean root tip temperatures (mean of 12 root tips temperatures during the corresponding interval) in all four species. Within each species, linear regressions between RER $12h^{-1}$ at night and root tip temperatures were always closer than regressions with RER $12h^{-1}$ day values (Fig. 4.4). For each mean root tip temperature we also presented the coldest and warmest hour during the 12 h interval (grey line in Fig. 4.4). Especially during the night interval, the warmest hour did not affect the mean root tip temperature, indicating by the skewed position of the mean on the grey line.

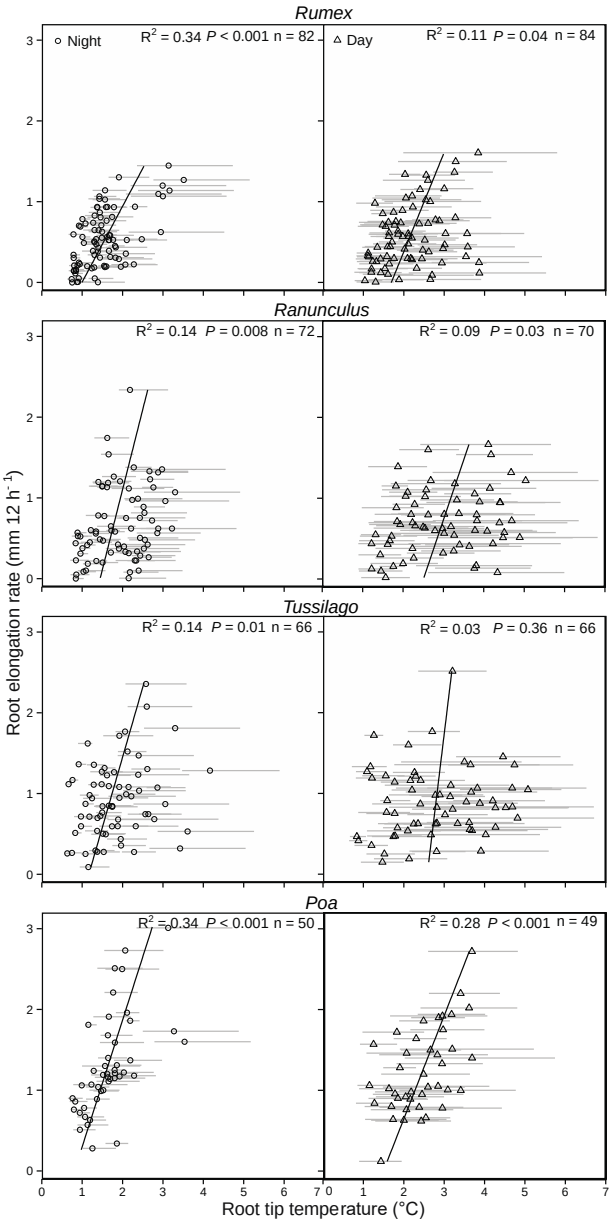
We were able to record very small RERs of less than 0.2 mm $12h^{-1}$ of a few individual roots between 0.7 and 1.2 °C during overcast days and cooler night intervals at ca. -60 mm soil depth (Fig. 4). However, comparing the RERs at these low temperatures between the four species indicates that species may vary substantially in their capability to elongate their roots at these low temperatures. For instance, *Poa* roots elongate 0.6 mm $12h^{-1}$ whereas the RERs of the three forbs were lower (Table 4.1).

As expected, RERs between 1.0 and 5 °C were always significantly lower than the RERs in the control ($P<0.001$ for all species, Table 4.2). No correlation was found between RERs and the small variation in root tip temperature in the warm control (data not shown).

Root length increments in the cold treatment became smaller with increasing soil depth but roots still continued elongating very slowly as they approached the low temperature RER threshold.

At harvest the temperatures at the maximum rooting depth obtained, were in the range of 0.8 to 1.4 °C °C for all four species, taking the hourly maximum temperatures during the root forming period into account (Table 4.1). In *Poa*, the single deepest root grew to 105.8 mm corresponding to a temperature of 1.0 °C with hourly minimum and maximum temperatures of 0.8 and 1.3 °C whereas the deepest roots of the other species were found between 74 and 87 mm (Table 4.1). Averaging the deepest single roots across several cylinders supported the difference between the three forbs and the grass species. *Rumex* roots stopped at higher soil depth, thus slightly warmer temperature, corresponding to 2.4 ± 1.1 °C, then *Ranunculus* at 2.0 ± 0.9 °C, *Tussilago* at 1.9 ± 0.8 °C and *Poa* at 1.5 ± 0.3 °C (Table 4.1). The roots of the 10 °C control all reached the bottom of the cylinders (180 mm).

Figure 4.4



Root elongation rates (RERs) during 12h intervals (right: day, left: night) in relation to temperature at root tip position (cold treatment only). Each dot presents the mean temperature and the line the range between hourly minimum and maximum temperatures during 12 h interval. Number of replicates: *Rumex*: n = 10 roots of 10 different individuals; *Ranunculus*: n = 9; *Tussilago*: n = 8; *Poa*: n = 7.

4.4.3 Root, leaf and plant traits

At harvest (after 29 full treatment days) total root length of cold treated plants, including first- and second-order roots, reached only 3% (*Rumex*), 9% (*Ranunculus*), 13% (*Tussilago*) and 10% (*Poa*) of the length of roots of the control plants (Table 4.2). Final root dry weight was similarly affected. In cold soils, the root systems were not only reduced in size (Table 4.2), but roots were significantly lighter per unit length that means reduced SRL m g^{-1} in all species. Low temperature almost completely inhibited the development of lateral roots, compared to the high number of secondary roots in the warm treatment (Table 4.2). If any, lateral roots were found only close to the root base in the warmer uppermost centimetre of the substrate (Fig. S4.4). The root mass fraction (RMF) was significantly lower in the cold substrate in all species (-66% across all species in comparison to the controls).

Leaf area (cm2), leaf dry mass (g), SLA (m^2 g^{-1}) and also the total above-ground dry weight (g) were significantly smaller and lower, respectively, in cold compared to warm soils except for *Ranunculus. Ranunculus* showed a slightly reduced leaf area in the cold but the leaf mass, SLA and total above-ground dry weight did not differ between the temperature treatments. Leaf mass fraction (LMF) was not affected by the temperature treatments. Unexpectedly, stem mass fraction (SMF) was higher in *Ranunculus* and *Tussilago* under cold treatment but slightly lower in *Rumex* and unaffected in *Poa* (Table 4.2).

Table 4.2: Root elongation rate (RER), root traits, shoot traits and biomass fractions (mean ±sd). P-values from one-way ANOVAs, testing trait as dependent variable between cold and warm treatment for each species. Significance differences at P < 0.001 '***'; P < 0.01 '**'; P < 0.05 '*'. SLR specific root length, SLA specific leaf area, BGB below-ground biomass, AGB above-ground biomass, LMF leaf mass fraction, SMF Stem mass fraction and RMF root mass fraction

	Rumex			*Ranunculus*			*Tussilago*			*Poa*		
	Cold	Warm	*P*	Cold	Warm	*P*	Cold	Warm	*P*	Cold	Warm	*P*
Below-ground												
RER (mm 12 h^{-1})	0.5 ± 0.4	4.4 ± 1.6	***	0.7 ± 0.4	2.5 ± 0.9	***	0.9 ± 0.5	3.2 ± 1.1	***	1.2 ± 0.5	3.5 ± 1.4	***
Total root length (cm)	26.6 ± 15.8	858 ± 267	***	19.6 ± 10.6	153 ± 66	***	42.4 ± 20.9	478 ± 223	***	46 ± 31	477 ± 198	***
Root dry weight (g)	0.01 ± 0.01	0.17 ± 0.06	***	0.03 ± 0.02	0.09 ± 0.04	***	0.02 ± 0.02	0.08 ± 0.04	***	0.02 ± 0.01	0.05 ± 0.02	***
SLR (m g^{-1})	19.1 ± 4.3	52.8 ± 10.3	***	8.31 ± 3.7	19.6 ± 7.9	***	23.5 ± 8.6	58.6 ± 7.2	***	27.8 ± 10.5	100 ± 22.6	***
N. of primary roots	9.4 ± 4.2	12 ± 2.8	0.06	5.6 ± 2.1	5.9 ± 1.8	0.71	8.7 ± 2.9	11.7 ± 5.8	*	5.3 ± 2.4	9.8 ± 3.1	***
N. of secondary roots	7 ± 8.1	1959 ± 557	***	6.7 ± 10.5	176 ± 73	***	19.2 ± 17.1	829 ± 436	***	33.7 ± 30.7	1344 ± 536	***
BGB (g)	0.87 ± 0.31	1.06 ± 0.29	0.08	0.09 ± 0.03	0.12 ± 0.05	*	0.14 ± 0.05	0.19 ± 0.04	**	0.08 ± 0.02	0.12 ± 0.03	***
Above-ground												
Leaf area (cm^2)	24.9 ± 7.95	74.8 ± 14.3	***	4.72 ± 1.89	5.86 ± 1.82	0.09	7.19 ± 1.87	19.3 ± 8.51	***	3.77 ± 1.29	8.33 ± 2.27	***
Leaf mass (g)	0.23 ± 0.07	0.39 ± 0.09	***	0.05 ± 0.02	0.06 ± 0.02	0.17	0.07 ± 0.02	0.12 ± 0.05	***	0.04 ± 0.02	0.06 ± 0.03	*
SLA (m^2 g^{-1})	11.4 ± 3.53	19.9 ± 2.97	***	9.44 ± 1.78	9.41 ± 0.93	0.95	10.9 ± 1.31	15.5 ± 2.14	***	9.09 ± 2.57	16.6 ± 9.79	***
AGB (g)	0.32 ± 0.11	0.52 ± 0.13	***	0.13 ± 0.05	0.11 ± 0.03	0.23	0.09 ± 0.03	0.16 ± 0.06	***	0.11 ± 0.04	0.15 ± 0.05	**
Biomass fractions												
LMF	0.68 ± 0.05	0.56 ± 0.05	0.12	0.33 ± 0.06	0.33 ± 0.08	0.23	0.57 ± 0.07	0.52 ± 0.05	0.23	0.38 ± 0.13	0.31 ± 0.05	0.44
SMF	0.25 ± 0.04	0.19 ± 0.02	0.07	0.49 ± 0.11	0.24 ± 0.09	**	0.26 ± 0.06	0.14 ± 0.03	**	0.47 ± 0.12	0.45 ± 0.06	0.62
RMF	0.04 ± 0.02	0.24 ± 0.07	***	0.16 ± 0.08	0.42 ± 0.11	***	0.17 ± 0.08	0.33 ± 0.05	***	0.14 ± 0.05	0.24 ± 0.03	**

4.4.4 Root anatomy

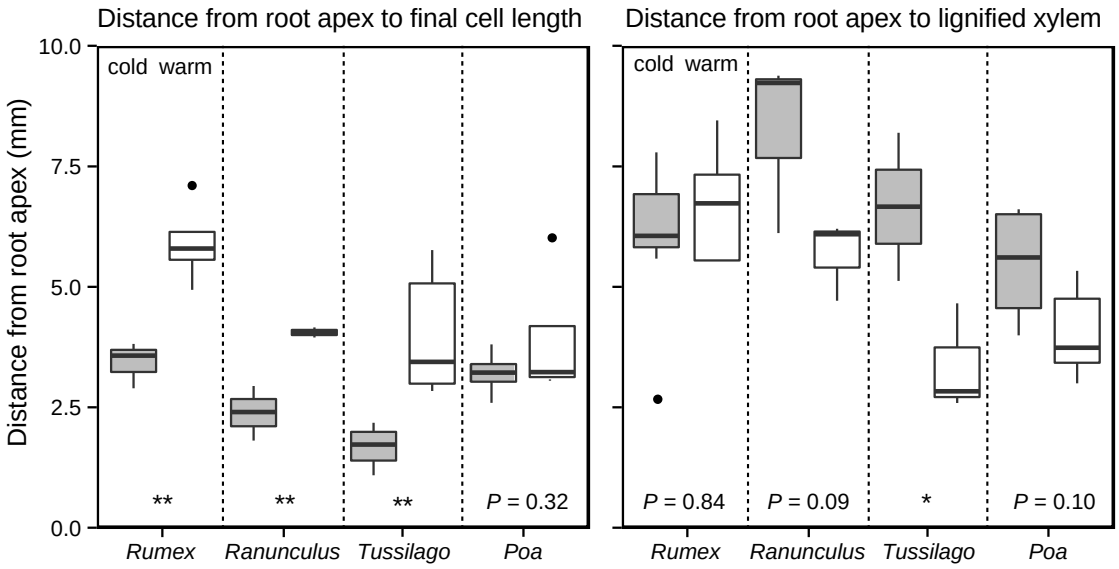
For each species, the deepest roots were selected for the anatomical assays. Nevertheless, the number of root replicates per species dropped as only perfectly longitudinal sections of roots were further processed. For *Poa*, the hourly maximum root tip temperatures of the selected roots were never higher than 2 °C, for *Rumex* (except one root), *Ranunculus* and *Tussilago* (except one root), root temperatures never surpassed 3 °C.

The cell density in the root tip, counted right after the root meristem initials (root apex) in an area of 0.2 x 0.2 mm was similar in cold and warm treatments (Table 4.3) and these meristematic cells had diameters between 8 and 10 µm. Unexpectedly, the low temperature had no significant effect on the final cell length in *Poa* and *Rumex*, but a trend to shorter cells was found in cold treated *Ranunculus* and in *Tussilago* (Table 4.3). The distance-to-apex data revealed that cell elongation was strongly reduced by low temperature in all three forb species, but not significantly in the grass species (Fig. 4.5). Thus, in cold treated roots, cells remained in the small (meristematic) state over a longer distance from the apex compared to warm treated roots, but counter expectation, reached final cell length over a shorter distance from the apex. Yet, there were much fewer elongating cells in the elongation zone than in the warm treated roots in which cells reached final cell length over a longer distance, and there were more of these elongating cells.

The staining with fluorescent berberine-aniline blue employed here, was effective to detect xylem lignification in the thin sections of the roots. Counterstaining with aniline blue inhibited any other fluorescence signals in the root tissues and, although berberine is not considered a lignin specific dye (Brundrett et al., 1988), it intensified the fluorescent signal of lignified xylem cell walls in contrast to non-lignified walls and allowed us to quantify the lignification optically. The low temperature treatment retarded the lignification of the xylem, indicating a lower rate of cell differentiation in the cold treatment. The first lignified xylem elements (fluorescent signal observed through light microscopy) emerged at a greater distance from the root apex in cold grown roots (Fig. 4.5). In *Rumex* roots, the species that stopped growing at relatively warmer temperature, the lignified xylem was detectable at a similar distance from the apex in both temperature treatments. The degree of lignification, measured as lightness (L-channel value) of the fluorescent xylem showed an overall higher sensitivity than the direct visual observation. The lightness values were significantly lower and remained lower with increasing distance from the apex under cold conditions for three species (trend only in *Ranunculus*;

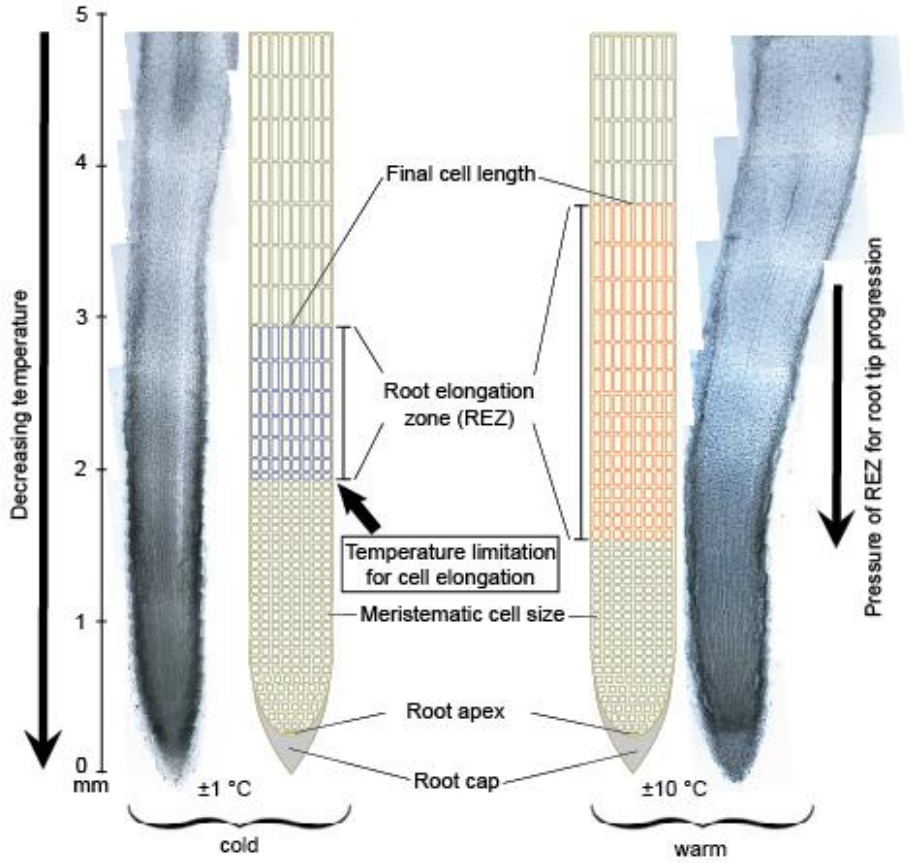
Fig. 4.7, Table 4.4). Both *Ranunculus* and *Poa* showed brighter fluorescence signals than the other two species, suggesting a higher degree of lignification compared to *Tussilago* and *Rumex* (Fig. 4.7, [Supplementary Information, Fig. S4.4]).

Figure 4.5



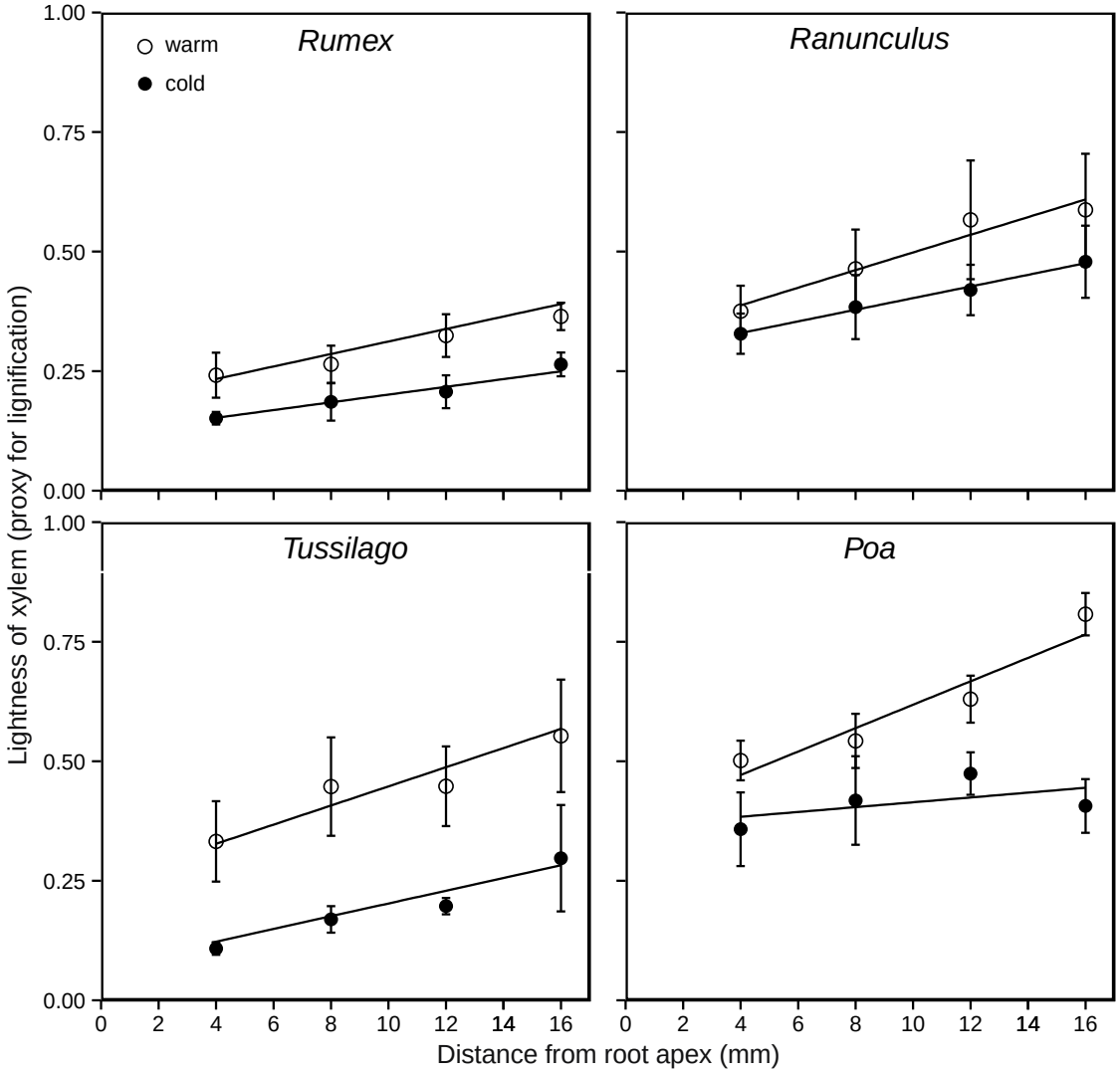
The distance between the root apex and the first cells reaching final cell length (left), and the distance between the root apex and the first lignified xylem elements (right; for number of replicates see Table 4.3).

Figure 4.6



Root tips (5 mm length) of *Tussilago farfara* are shown as an example to visualize the effect of a shortened root elongation zone under low temperature. Due to the vertically decreasing temperature, cell elongation to final length was inhibited when roots reached a certain soil depth, that means lower temperature.

Figure 4.7



Lightness indices (mean ± sd; L-channel of the HSL colour space) as a measure for lignification of xylem elements in 4 mm steps along the terminal 20 mm of the root tips (for number of replicates, see Table 3 and for the linear models, Table 4).

Table 4.3: Cell density in an area of 0.2 x 0.2 mm close to the root apex and the final cell length reached in >5 mm distance from the root apex (mean ± sd). Number of replicates (from different individuals): *Rumex* n = 4, *Ranunculus* n = 5, *Tussilago* n = 6 and *Poa* n = 6

	Rumex		*Ranunculus*		*Tussilago*		*Poa*	
	Cold	Warm	Cold	Warm	Cold	Warm	Cold	Warm
Cell density close	353 ± 44	342 ± 20	332 ± 18	351 ± 15	313 ± 25	323 ± 20	400 ± 39	400 ± 18
Final cell length	0.21 ±	0.23 ±	0.18 ±	0.24 ±	0.14 ±	0.19 ±	0.31 ±	0.28 ±

[#] Trend for shorter cells in *Ranunculus* (P=0.09) and *Tussilago* (P=0.06) under cold treatment

Table 4.4: Treatment effect on xylem lignification (L-values). Linear models for treatment (warm vs. cold) and distance from root apex (nested design; model for each species)

Species	Factor	Estimate	Std.	t value	P value
Rumex	Intercept	0.21	0.03	6.6	<0.001
	treatment	0.12	0.02	2.9	<0.01
	distance	0.01	0.01	3.4	<0.01
$R^2 = 0.46$					
Ranuculus	Intercept	0.35	0.07	5.1	<0.001
	treatment	0.25	0.05	4.3	0.076
	distance	0.02	0.01	2.7	0.014
$R^2 = 0.27$					
Tussilago	Intercept	0.29	0.07	4.3	<0.001
	treatment	0.04	0.05	0	<0.001
	distance	0.02	0.01	2.8	0.011
$R^2 = 0.57$					
Poa	Intercept	0.46	0.06	8.4	<0.001
	treatment	0.26	0.04	3.7	<0.001
	distance	0.02	0.01	3.2	<0.01
$R^2 = 0.46$					

4.5 Discussion

We grew four alpine taxa under typical alpine climate for above-ground plant tissues, but at two tightly controlled, contrasting root zone temperatures. The employed water bath systems combined with effects of solar radiation on the top of the soil column induced a sharp vertical temperature gradient in the cold treatment. Temperature differences in the top 30 mm where the ambient climatic conditions had a strong influence were substantial but below that level, temperatures decreased sharply with increasing soil depth in the cold treatment. At soil depths between -40 and -60 mm, where most cold treated roots reached the cylinder walls, temperatures were mainly below 5 °C (Fig. S4.2). Lowest tip root temperatures during RER measurements were 0.7 to 1.2 °C and roots of all four alpine species were still capable to elongate at these low temperatures.

To delineate the physiological minimum temperature for root growth (*sensu*: root formation) we took the single deepest root per species formed during the experiment and that root position corresponded to 1.0 °C in *Poa alpina* and between 1.0 - 1.2 °C for the three forb species taking the hourly maximum temperature during the period of the single deepest root formation into account, the critically low temperature for root growth is 1.4 °C (Table 4.1).

Given that one single deepest root may not fully represent the minimum temperature threshold for the species, we calculated a mean of more than 20 single deepest roots per species and their corresponding root temperatures. *Poa* had the lowest mean temperature threshold with 1.5 °C, followed by 1.9 °C in *Tussilago farfara*, 2.0 °C in *Ranunculus glacialis* and 2.4 °C in *Rumex alpinus.* However the hourly maximum temperatures for this mean per species covered a much higher temperature range since not all deepest roots per cylinder reached the depth beyond -60 mm where temperature fluctuations became small (Table 4.1).

The temperatures thresholds for root growth observed here are slightly lower than those reported for montane tree taxa (Schenker et al. 2014), arctic plants (Bliss 1956; Ellis and Kummerow 1982) and our previous estimates based on longer root observation intervals (4 days) for *Ranunculus* and *Poa* (Nagelmüller et al. 2016a), which arrived at thresholds close to 2 °C for these two species.

The root systems that developed in the cold soil profiles were highly retarded and also AGB was negatively affected compared to controls, except for *Ranunculus*, showing

reduced leaf area but not significantly lowered leaf mass. Biomass allocation expressed as SMF and RMF was reduced under the cold treatment, except LMF. Unexpectedly, SMF was significantly higher in *Ranunculus* and *Tussilago* under the cold treatment.
The higher SMF could be an effect of delayed leaf unfolding, causing petiole mass to contribute to the higher stem fractions (in terms of their function, petioles were considered to belong to the stem fraction). Compared to the earlier in situ experiment with the species *Ranunculus* and *Poa* (Nagelmüller et al. 2016a), the low temperature treatment was more severe here, which contributed to the significantly negative effects on AGB that was not observed in that earlier work.

It is a crucial issue in studies that aim at defining thermal thresholds for growth that temperatures do not fluctuate and temperature sensors embody the needed temporal and spatial resolution as well as the accuracy. Temperature means may be fully misleading, particularly, if they include periods with warmer temperatures. In the present study, we always considered the hourly maximum temperatures during the corresponding observation period (RER and root formation period, respectively).

Counter expectation, the cell elongation zone in root tips was not longer but shorter in cold grown roots as we measured a shorter distance from the root apex to the first fully elongated cells (Fig. 4.6). We explain this observation by the fact that the sharp temperature gradient in the cold treatment did not permit root tips to expand beyond a certain temperature. Similar observations emerged in *Arabidopsis thaliana* roots exposed to 4 °C: root apices were deformed and growth zones shortened, causing a swelling in the primary roots (Plohovska et al. 2016). These authors explained the negative effect of low temperature on root elongation to be associated with impaired organization of the cytoskeleton, particularly microfilaments. Also in an earlier study on roots of three cultivars of winter wheat which differed in frost tolerance, Abdrakhamanova and co-workers (2003) related the changes in the microtubuli organization (especially, the disassembling of microtubuli during frost events) to the capability of roots of the tolerant cultivar to recover from frost and to grow at 4 °C.
We also assume feedback regulation from the elongation zone to the meristematic zone that causes a cessation of further cell production at such minimum temperature well known from root growth kinematic studies (Silk 1992, Beemster and Baskin 1998) and more recent work (Rost 2011, Kumpf and Nowack 2015). Interestingly, the most low temperature tolerant species in terms of root elongation and rooting depth (and the only monocot), *Poa alpina*, almost retained the proportion between the length of the cell elongation zone and the meristematic zone.

The higher sensitivity of cell elongation compared to cell division at extremely low positive temperature causes the pressure for root tip progression into deeper (and colder) soils to cease and thus, reduces RER close to zero. The temperature limit for cell elongation can be expected to occur at slightly higher than the minimum temperatures estimated from root tip position (Table 4.1) because the end of the elongation zone was between 2 and 4 mm above the tip, corresponding to a ca. 0.1 - 0.2 K higher temperature. While cell elongation was clearly limited, the root apical meristem was still able to produce new cells, which accumulated for a longer distance from the apex and stayed small (in meristematic size). In the warm treatment, root cells at a similar distance from the apex kept elongating to final cell length (Fig. 4.6). These findings are in line with results of earlier studies that showed that growth restriction at low temperature does not start with an inhibition of cell division and cell production (Francis and Barlow 1988, Körner and Pelaez-Menendez-Riedl 1989). Yet, at some point (in time and/or space), cell production must be down-regulated through feedback from limited cell elongation and cell differentiation.

We assume that the limitation of cell enlargement and the associated cell differentiation process are critical for root growth at very low temperature. Xylem differentiation may play an important role and lignification is a potentially critical candidate. The lignification of conduits is essential for the functionality of the xylem, and only a tight conduit system can contribute to the needed turgor pressure required for soil penetration. In the low temperature treatment, lignified, and thus functional xylem vessels, became visible only at a greater distance from the root apex, and the lignin signal was less intense, although these cells had more time (in the sense of tissue development) to accumulate lignin in the cell walls. The limited lignification of cold grown roots may contribute to the fragility and overall 'glassy' texture of the whitish root tissues produced below 5 °C. Similar morphological changes in cold treated roots were reported by Schenker et al. (2014) and Nagelmüller et al. (2016a). However, the biochemical processes underlying the inhibited lignification are still unclear, especially, whether the synthesis of lignin and its precursors, and/or the lignin deposition (polymerization) are affected under cold temperature awaits a further explanation. Cold acclimation in plants (to chilling, positive temperatures) has often been associated with increased lignin contents in different plant organs including roots (Cabane et al. 2012 and citations therein). On the other hand, Donaldson (2001) reported that lignification of the secondary wall of latewood tracheids was often incomplete at the onset of winter, thus suggesting that lignification is sensitive to temperature. The lower temperature threshold for

xylogenesis in the alpine *Rhododendron* shrub (2.0 ± 0.6 °C) than in conifers at the treeline (4-5 °C; Rossi et al. 2008) has been interpreted as a consequence of exposure to cooler microclimate of the alpine shrub (Li et al. 2016), particularly, at nights when radiation losses are high and convective heat exchange is low. The critically low temperature for lignification in the alpine herb and grass species observed here corroborated this minimum temperature and may be a common threshold for lignification in many cold adapted angiosperms.

4.6 Supplementary information

Supplementary information is available online at https://academic.oup.com/aob and in the supplementary material of this thesis. Figure S4.1: frequency distribution of the hourly soil temperatures in the six sensor depths in the four baths (three cold and one warm control bath) during the 29 treatment days. Figure S4.2: the temperature course of the seven temperature sensors in the cold treatment cylinders (bath 2) during the period of root elongation measurements (RER). Figure S4.3: estimation of mean root tip temperature for a RER 12h-1 from soil depth and soil temperatures (based on polynominal regressions). Figure S4.4: snap shots of longitudinal cuts of warm and cold treated root tips showing the reduced lignification.

4.7 Acknowledgments

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5 Critically low soil temperatures for root growth and root morphology in three alpine plant species*

***** This additional manuscript was written during the time of the doctoral thesis with data from the previously conducted master thesis. The results of this paper were published in the journal "Alpine Botany" and gave promising insights into the cold temperature limitations of root growth in alpine plants, which were decisive for the development of ideas and the realization of this doctoral thesis.

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Keywords
Alps, biomass allocation, high elevation, meristems, mountains, root elong

5.1 Abstract

Limited rates of tissue formation most likely constrain plant growth at low temperatures. Yet, temperature thresholds and growth dynamics under critically cold conditions have still to be identified. We utilized microhabitat contrasts in soil temperature along glacial and melt water streams to study temperature effects on root extension growth and root morphology of *Ranunculus glacialis*, *Homogyne alpina* and *Poa alpina* at ca. 2500 m elevation in the Swiss Alps. Plants were grown in containers filled with glacial silt, embedded in stream water, with root windows allowing the monitoring of root development for 41 days in naturally 'cold' substrate temperatures (hardly ever exceeding 5 °C), and 5-8 K warmer substrates. Plants grown in the cold substrate had significantly less roots and roots were pale and unbranched. Under cold conditions, total root length was 83% shorter and total root dry mass was 67% lower than in warm substrate. In contrast, aboveground biomass was hardly affected. Mean root elongation rates were 47% lower under cold substrate conditions. The frequency distribution of root temperatures and root elongation rates showed a substantial reduction of apical root growth below 3-5 °C, with growth approaching zero at ca. 2 °C. A thermal sum of ca. 20 °h >2 °C per four-day interval is at least required for detectable root elongation, At a 5 °C threshold, thermal sums of ca. 40 °h >5 °C within four days allowed the formation of a fully developed root system during the short alpine summer. Substrate temperatures above 10 °C exerted no further stimulation in root growth.

5.2 Introduction

Temperature is a major driver in regulating growth and distribution of plants in mountains, and critically low soil temperatures have been shown to play an important role for growth and productivity in both, temperate- and arctic-alpine plants (Peacock 1975, Baker and Younger 1986, Chapin 1983, Körner 2003). Temperature thresholds co-control the initiation of new growth (Billings and Mooney 1968), but also constrain the rate of tissue formation during the growing season (Alvarez-Uria and Körner 2006, Schenker et al. 2014). Driven by topography, alpine landscapes embrace a variety of soil temperatures ranging from very cold to warm conditions over short distances only (Scherrer and Körner 2010, Wundram et al. 2010). This offers the possibility to explore the influence of cold temperature on tissue formation *in situ*, the central aim of this study.

Root and shoot growth are assumed to exhibit similar temperature responses, given that all meristems are expected to employ the same processes at cell and tissue level. Cold adapted plant species generally have a lower temperature optimum for growth (Chapin 1983), but also lower threshold temperatures for minimum growth (Woodward et al. 1986). The consequence of a relatively high growth rate at low temperature is most likely related to a lower maximum rate of growth (Körner and Woodward 1987). It has been hypothesized that all cold adapted plants face very similar temperature related growth limitations (Körner 2006, 2008). Thus, cold adapted lowland plants such as winter crops display the same temperature threshold for tissue formation as cold adapted trees at treeline, showing appreciable growth only when temperature close to the meristems rose above 5 °C (Rossi et al. 2007, Alvarez-Uria and Körner 2007, Körner 2008). Below 5 °C, rates rapidly approach zero. For the alpine grass *Poa alpina* leaf elongation rates reached zero near the freezing point (Körner and Woodward 1987). Winter wheat and winter barley showed minute leaf growth rates at 5 °C and rates were extrapolated to zero growth at 2 - 3 °C (Gallagher et al. 1979, Gallagher and Biscoe 1979). Similarly, leaf expansion in sugar beet ceased close to 5 °C (Milford 1984). Roots in evergreen and deciduous tree species revealed similar low temperature limits for growth, with the bulk of the roots produced at temperatures above 5 °C, but an absolute temperature limit for root growth of deciduous tree species at 2.3 °C (Schenker et al. 2014). Since the decline in growth at cold temperatures is asymptotic, absolute thresholds may be arbitrary and it also depends on the length of the observation interval whether they become detectable.

The causes of the growth limits at low temperature are still poorly understood. Using the metaphase arrest method, the mitotic cycle of cold adapted plants was found to be robust against low temperatures (Francis and Barlow 1987, Körner and Pelaez-Menendez-Riedl 1989, Körner 2003), hence cell and tissue differentiation are most likely decisive and feeding back on the cell cycle, rather than the other way round (Körner 2003, 2008). Cold adapted plants maintain 50 - 70% of their photosynthetic capacity at leaf temperatures of 5 °C and still c. 30% of the maximum photosynthetic activity at 0 °C (Tieszen 1975, Körner 2003). Cell wall formation and building watertight xylem conduits (i.e., lignification) are therefore among the most critical processes, rather than the rate of assimilate provision (Körner 2003, 2012).

This study aims at testing the impact of low soil temperatures on root growth (root elongation and morphology) in alpine plants under otherwise natural growth conditions. We also analysed leaf area, specific leaf area and aboveground biomass in response to the contrasting root temperatures. The studied three alpine species were grown next to each other under either ‘warm’ or ‘critically low’ substrate temperatures. For this manipulative experiment, we utilized temperature differences shaped by glacial and melt water streams at around 2500 m a.s.l. in the Swiss Alps.

5.3 Material and Methods

5.3.1 Experimental site and plants

The experiment was carried out near the ALPFOR research station, close to the Furka Pass, at three sites between 2440 and 2550 m a.s.l. in the Swiss central Alps. Within each site, a cold and a warm location was selected.

Small, similarly sized individuals of the two forb species *Ranunculus glacialis* L. (Ranunculaceae), *Homogyne alpina* (L.) Cass. (Asteraceae) and the grass species *Poa alpina* ssp. *vivipara,* L. (Poaceae) were collected at a very early seasonal developmental stage in the vicinity of the test sites shortly after snowmelt. *Ranunculus glacialis* is a representative species for the glacier forefield, whereas *Poa alpina* spp. *vivipara* also occurs -besides its abundance in glacier forefields- in subalpine and alpine grassland (e.g, Steiner et al. 1997). In glacier forefield, the pseudo-viviparous form of *Poa alpina* dominates, producing plantlets out of the spikelets instead of seeds. *Homogyne alpina* is a typical species of late successional subalpine and alpine grassland. In the following, we address plant species by genus only.

All roots from the previous season were cut to a length of ca. 3 cm before planting, so, new roots could be clearly identified.

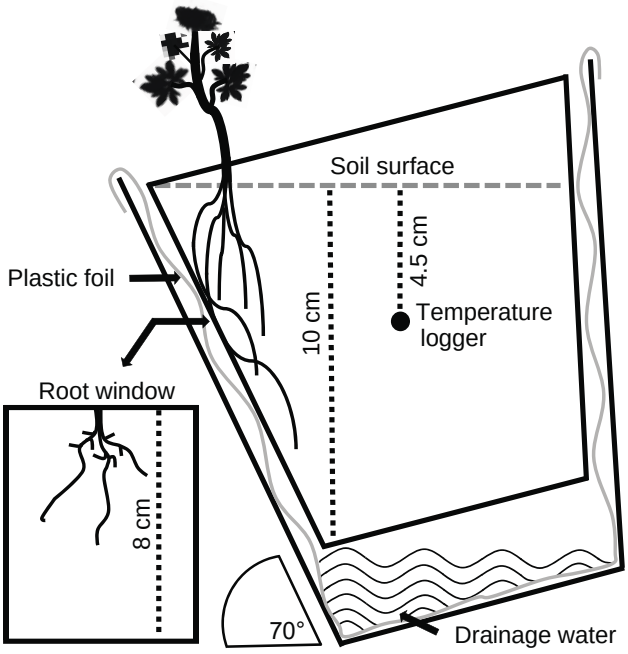
5.3.2 Experimental setup

The study plants were planted on the day of sampling in containers (Pflanzkorb V 1L, 10 x 10 x 10 cm, Gardena, Germany) filled with sandy glacier silt collected near the experimental site. The pH of this substrate was 6.9 ± 0.8 (mean ± sd), measured for nine soil samples, using 6 g dry soil mixed with 15 ml 0.01 M $CaCl_2$ and a portable pH meter (Russell RL060P, Thermo, Germany; and a gel-electrode Hügli Labortec, Abtwil, Switzerland). We applied 88 mg fine powdered slow-release inorganic fertilizer (H. Gilgen, Optima Werke, Münchenstein, Switzerland) to each container to avoid possible growth constrains due to nutrient limitation. The amount of fertilizer corresponded to 15 kg N ha^{-1} a^{-1}, which is two to three times the current, wet annual nitrogen deposition in the Swiss central Alps (Hiltbrunner et al. 2005).

Containers had a plastic window (8 x 8 cm) for root observations that was fitted into the side wall. Containers were tilted by an angle of 20° to the surface to force roots to grow along the plastic window. To facilitate the inspection of root windows, containers were inserted into a second container. Because part of the containers was embedded in

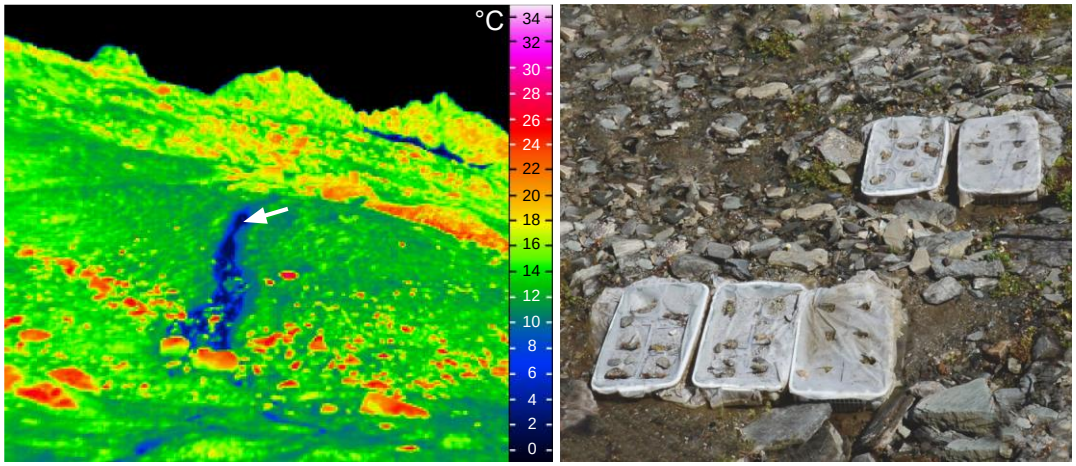
cold streams, plastic foil was placed between the two containers to prevent the inner one from becoming waterlogged (Fig. 5.1). Containers were assembled in groups in plastic baskets (30 x 50 cm) and embedded in sand (Fig. 5.2). We used eight plant replicates per species, site and temperature location (8 x 3 x 2 = 48 individual plants per species in total). For root elongation measurements, five containers per location had a plastic window only (n=5). Pairs of cold v*ersus* warm locations were replicated three times (nested design, i.e. location nested under cold and warm temperature regime, respectively). Cold substrate conditions were provided by installing baskets in small glacial or melt water streams, with cold water constantly perfusing the outer basket space, a situation not uncommon for glacier forefield plants, *R. glacialis* in particular. Warm-treated plants were placed at nearby drained, and thus, warmer locations (Fig. 5.2). Locations were named C1, C2, C3 for cold and W1, W2, W3 for warm locations, respectively. Substrate temperatures were monitored in one hour intervals in 4.5 cm soil depth in one container per location using single channel data loggers (Tidbit, Onset Computer Corporation, Bourne, MA, USA). Air temperature (1.4 m above ground) was recorded close to ALPFOR research station by a weather station (Vantage Pro2 Plus; Davies Instruments, Hayward, CA, USA). Soil moisture was measured weekly during the experiment as volumetric water content (% vol.) in five containers per location, using embedded moisture sensors (EC-10 with a handheld read out unit, Decagon Devices, Pullman, USA). Signals were standardized by the gravimetric soil water content in 100 cm^3 soil (n = 5 containers per location, dried at 80 °C) after plant harvest.

Figure 5.1



Design of plant container with root window (8 x 8 cm)

Figure 5.2



Experimental area in ca. 2500 m a.s.l. in the Swiss Alps. Left: Thermal image of the cold location (C1) with a cold water stream to generate cold substrate temperature. Arrow points to the position of plant containers. Right: Plant containers, outer containers in white boxes were perfused by cold water.

5.3.3 Root elongation measurements

Length measurements of first-order roots were taken in *Ranunculus* and *Poa* in four-day intervals. However, during first days, roots apparently did not reach the root window, hence there were in total eight intervals with root elongation rates per location covering a measuring period of 32 days and all containers were exposed for a duration of 41 days (till the final harvest). Root apex positions were marked on the plastic window and the increment was measured with a digital caliper (Mitutoyo, Urdorf, Switzerland). When several roots were growing along one root window, root length increments were averaged per container. Root growth could not be monitored in *Homogyne* (roots did not reach the root window).

5.3.4 Total root length and root morphology and biomass allocation

After 41 days plants were harvested at peak biomass, washed and photographed. Plants were separated into roots, rhizomes, leaves, and stems including flowers. Fractions were scanned with a flat bed transmitting light scanner (Epson Expression 1680, Epson, Meerbusch, Germany). Total root length, number of primary and lateral roots were calculated from scans, using the WinRHIZO software (Regent Instruments INC., Quebec, Canada). Dry weight was obtained after drying at 80 °C for at least 48 hours. Specific root length (SLR) and specific leaf area (SLA) were calculated by dividing the total root length (m) and leaf area (cm^2) by the corresponding dry weights (in g). For biomass allocation, root mass- (RMF), shoot mass- (SMF, including flowers) and leaf mass fractions (LMF) were calculated by dividing the dry weights of the fractions by the total plant weight (g).

5.3.4 Data and statistical analysis

One individual in *Ranunculus* (in C1), two in *Poa* (in C1 and C2) and seven in *Homogyne* (one in each of the three cold and four in W1) died during the experiment. *Homogyne* did not develop sufficient amounts of new roots in C1, thus only aboveground plant biomass and leaf area could be obtained in this location. Besides absolute minimum, mean and maximum temperatures (°C), temperature sums, i.e. degree hours (°h) above a defined temperature were calculated for the 41 days of the experiment and for each of the four-day census intervals. Degree hours (°h) above >2 °C, >3 °C and >5 °C were calculated by summing all hourly temperatures above 2 °C, 3 °C and 5 °C, respectively.

Differences in temperature variables between warm and cold locations were tested by two-sample t-test (pooled variance). Root elongation per census interval in *Ranunculus*

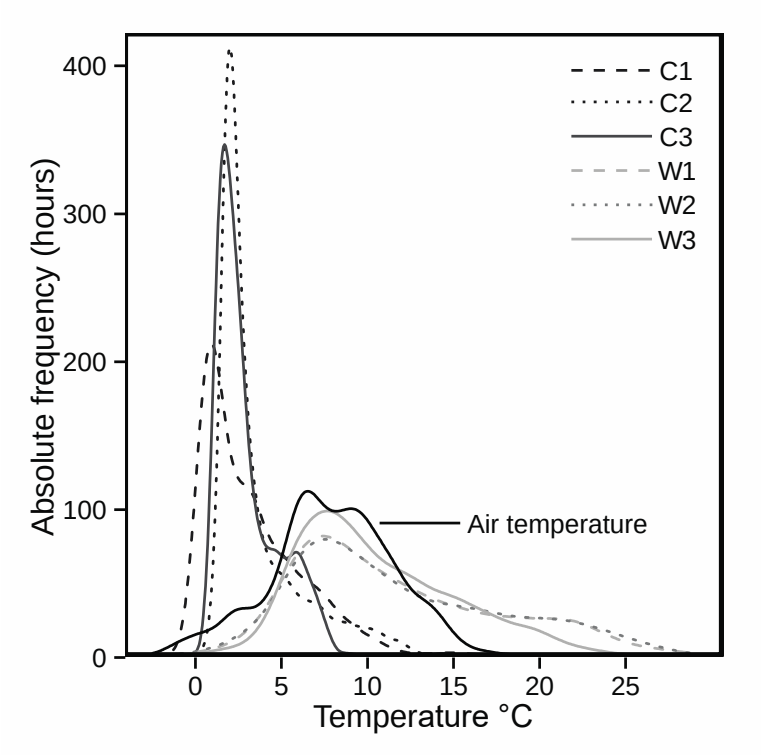
and *Poa* were regressed against the corresponding mean temperature and degree hours (°h >2 °C, °h >3 °C and °h >5 °C) by linear correlation. Dependent variables (total root dry weight, SRL, RMF, number of second roots, above- and belowground biomass, LMF, SMF and SLA) were tested for differences in temperature regime and locations (nested design) by analysis of variance followed by a posteriori Tukey-Kramer HSD test. Values of all dependent variables were log-transformed to achieve normal distribution. All statistical analyses and diagrams were performed with R (version 3.0.2; R Development Core Team 2013) and the package "ggplot2" (Wickham 2009).

5.4 Results

5.4.1 Substrate temperature and moisture

The 41-day mean temperature in 4.5 cm substrate depth at the three cold locations (3.5 ± 2.4 °C) was on average 7.9 K colder than in the warm locations (11.4 ± 5.5 °C; t = 17.2, P < 0.001). Absolute maximum temperatures and degree hours (°h >2 °C, °h >3 °C and °h >5 °C) were also significantly smaller in cold locations, while absolute minimum temperatures were equally low as a result of short chilling weather events. Under cold conditions degree hours added up to 1354 ± 264 °h >2 °C, 907 ± 244 °h >3 °C and 393 ± 186 °h >5 °C, under warm to 8662 ± 685 °h >2 °C (t = 17.2, P <0.001), 7746 ± 686 °h >3 °C (t = 16.3, P <0.001) and 5959 ± 702 °h >5 °C (t = 13.3, P <0.001) for the entire 41 day observation period (10 and 15 times higher temperature sums in the warm treatment; Tab. 1). As indicated by the mean temperatures and the different degree hours, temperatures at cold locations were mostly below 5 °C (Tab. 1, Fig. 5.3). The right tail of the frequency distribution indicates that the cold treated root zones (especially at C1 and C2) reached brief episodes of maximum soil temperatures of 10 °C during hours with strong solar radiation (in total, 36 hours >8 °C during 41 days). The temperatures in the warmer locations varied over a larger range, but were almost always above 5 °C (Fig. 5.3). Mean substrate moisture was always high (mostly above >35 % vol., Tab. 5.1) but slightly lower at warm locations. Containers were watered whenever moisture dropped below 25 % vol. (Tab. 5.1). The temperature treatment had an effect on phenology, so that flowering started on average 15 days later at the cold locations and the pedicel was only half of the length of plants grown in warm substrate at plant harvest.

Figure 5.3



Frequency distribution of root zone temperatures in 4.5 cm substrate depth and air temperature (weather station in 1.4 m height; hours in classes of 0.1 °C). C1-C3: cold locations, W1-W3: warm locations

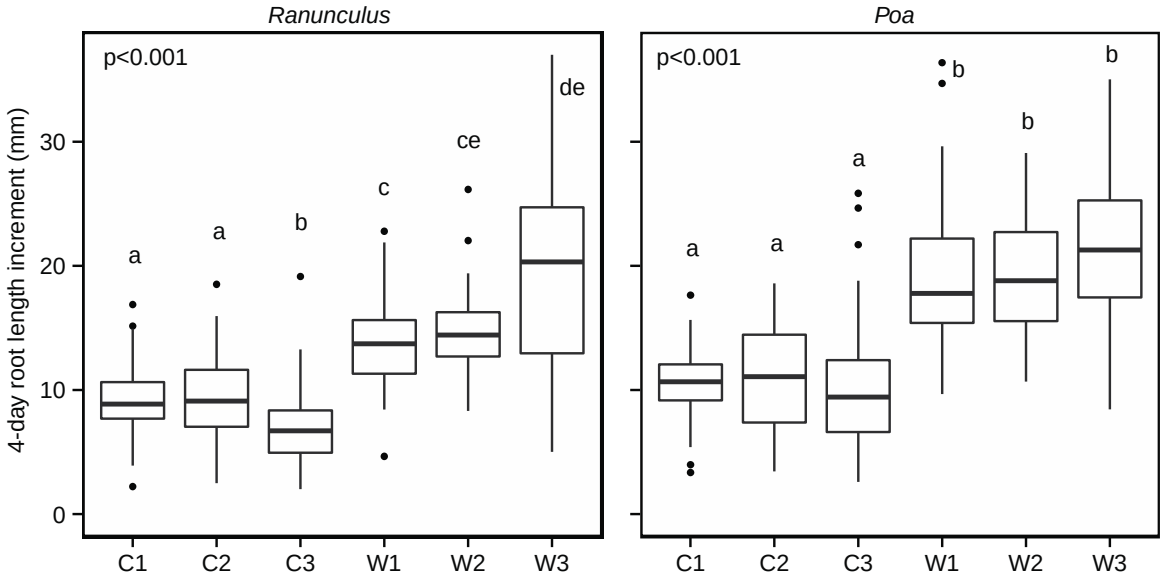
Table 5.1: The temperature regime in the rooting zone during the experiment at 3 warm (W1-W3) and cold (C1-C3) locations close the Furkapass. Absolute minimum, absolute maximum refer to single lowest and highest hourly temperature during 41 days, °h >2 °C, °h >3 and °h >5 °C refer to degree hours of substrate temperatures (°C) in 4.5 cm soil depth. Mean soil moisture (% vol.) from weekly measurements

Site	C1	C2	C3	W1	W2	W3	Air
abs. min T	0.05	0.88	0.83	0.85	0.88	1.21	-1.33
mean T ± sd	3.1 ± 2.7	3.5 ± 2.4	2.9 ± 1.8	11.7 ± 5.8	11.9 ± 6	10.5 ± 4	7.9 ± 3.5
abs. max T	15.65	12.82	7.87	28.12	27.45	23.54	17.27
°h >2 °C	1533	1480	1051	8962	9147	7878	5562
°h >3 °C	1079	1015	627	8046	8232	6961	4709
°h >5 °C	494	507	178	6263	6458	5156	3098
Soil moisture	39 ± 1	38 ± 1	39 ± 1	32 ± 3	35 ± 2	38 ± 1	

5.4.2 Rates of root length increment

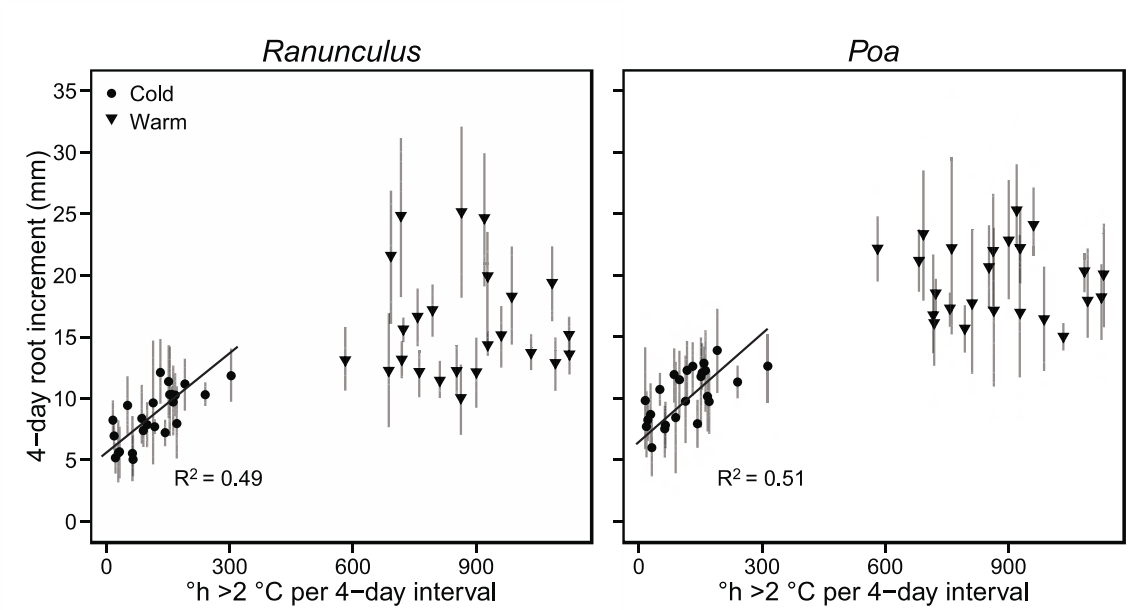
Roots of *Ranunculus* and *Poa* continued elongating throughout the experimental period in both cold and warm substrates. Root elongation was significantly slower in both species under cold conditions. Within the cold locations, *Ranunculus* roots elongated less in C3 than in the other two cold locations C1 and C2 (Fig. 5.4). Overall the thinner roots of *Poa* showed a slightly faster root length increment than the thick roots of *Ranunculus* (Fig. 5.4). Mean root length increments were linearly and positively related to mean substrate temperatures for both species under the cold treatments (*Ranunculus*: $R^2 = 0.49$; $P < 0.001$ and *Poa*: $R^2 = 0.54$, and; $P < 0.001$). No such correlation was found in the warm substrates, hence, the variation of the generally warmer temperature did not affect root elongation rates. Similarly positive correlations were found when testing the mean root length increments against °h > 2 °C (*Ranunculus*: $R^2 = 0.49$, $P < 0.001$ and *Poa*: $R^2 = 0.51$, $P < 0.001$, Fig. 5), °h >3 °C (*Ranunculus*: $R^2 = 0.46$, $P < 0.001$ and *Poa*: $R^2 = 0.36$, $P < 0.01$) and °h >5 °C (*Ranunculus*: $R^2 = 0.36$, $P < 0.001$and *Poa*: $R^2 = 0.25$, $P < 0.05$) at the cold locations. Besides the temperature means, sums of ° h >2 °C yielded the best fit (i.e., the closest relationship) to the mean root length increments in both species (Fig. 5.5).

Figure 5.4



Boxplot of the four-day root elongation rate in *Ranunculus* and *Poa* for each location over the 32-day census duration (8 intervals, n = 5 replicates per location). Boxes comprise the 25 - 75 % quantile, horizontal line represents median. Dots indicate outliers. P-values indicate significant difference between temperature regime and different letters between locations (nested design, posteriori Tukey HSD, P<0.05). C1-C3: cold, W1-W3: warm locations.

Figure 5.5



Correlation between 4-day root increments (mean ± se, n = 5 per location) and degree hours (°h) >2 °C, measured in 4.5 cm substrate depth. The variation in temperature is due to variable weather conditions. Linear correlation within cold locations: *Ranunculus*: $R^2 = 0.49$, $P < 0.001$; *Poa*: $R^2 = 0.51$, $P < 0.05$. There was no correlation between mean root increment and degree hours within warm locations.

5.4.3 Root morphology, total root length and aboveground biomass

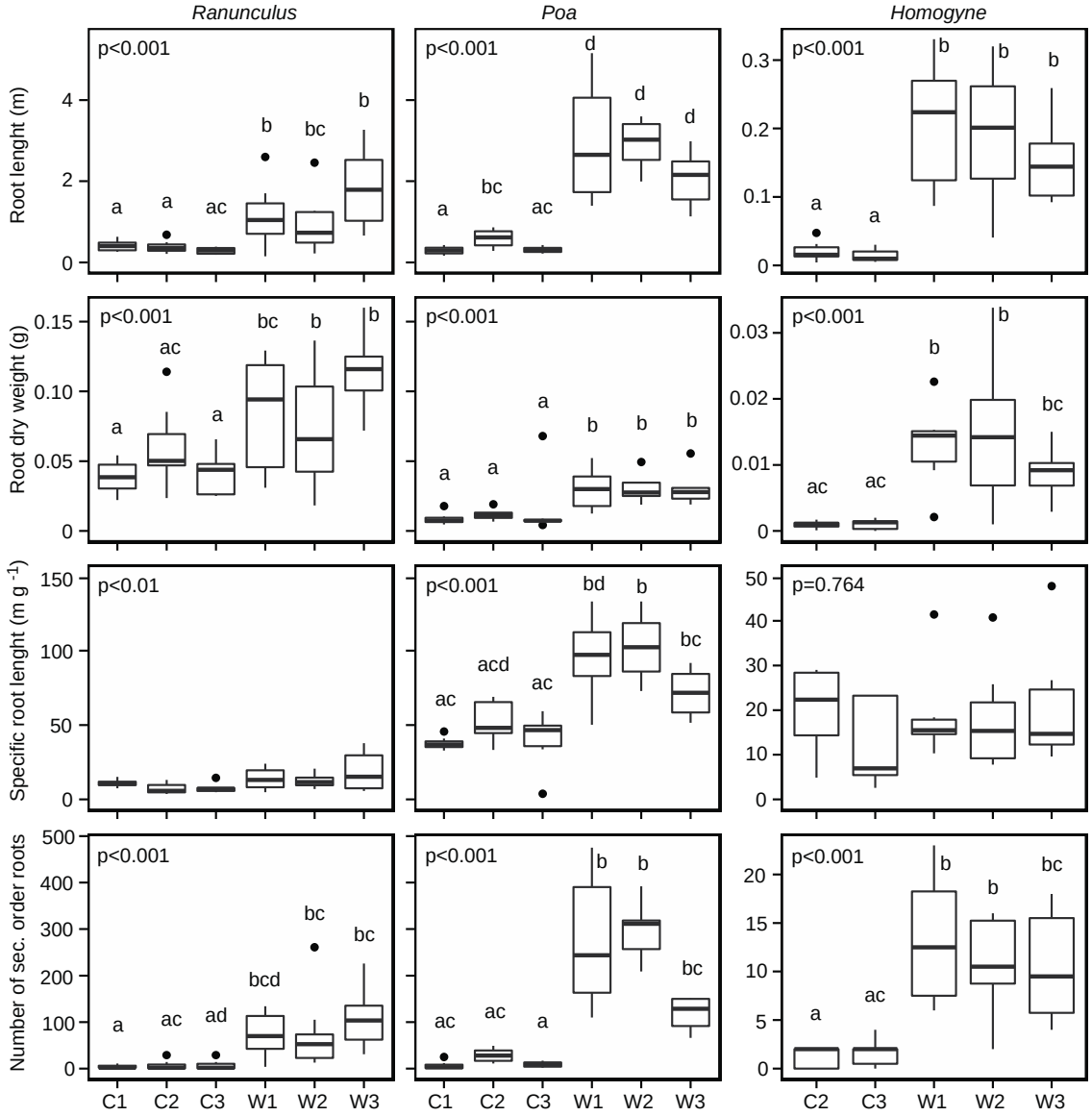
At the time of peak biomass, the three cold treated plant species had smaller root systems. There was little variation in root length and weight among cold locations, whereas these root parameters showed a higher variation and were on average substantially larger in plants grown under warm conditions (Fig. 5.6). In comparison to the other two species, *Homogyne* developed much less roots and, as mentioned above, produced hardly any roots at location C1, however, total root weight was also lower in this species under cold conditions. In the cold, roots of all three species were almost unbranched (i.e., no or low number of second-order roots), shorter and pale in colour (Fig. 5.6, Fig. 5.7). In addition, cold grown roots had a glassy texture, while warm grown roots were typically brownish and also less fragile. The few second-order roots were very short and confined to the uppermost centimeter of the substrate where the cold treatment was not so intense.

Total root length, including first and second-order roots, was on average shorter in cold compared to warm substrates: in *Ranunculus* by 73%, *Poa* 85% and *Homogyne* 91%. Correspondingly, root dry weight and total belowground biomass (including rhizomes) was on average 67%, respectively 28%, less in cold compared to warm substrates across

species (Fig. 5.6, Tab. 5.2). Differences in specific root length SRL between cold and warm locations were less pronounced except for *Poa*, showing a significantly lower SLR at cold locations (Fig. 6). RMF was always significantly lower at cold locations in all three species (Tab. 5.2).

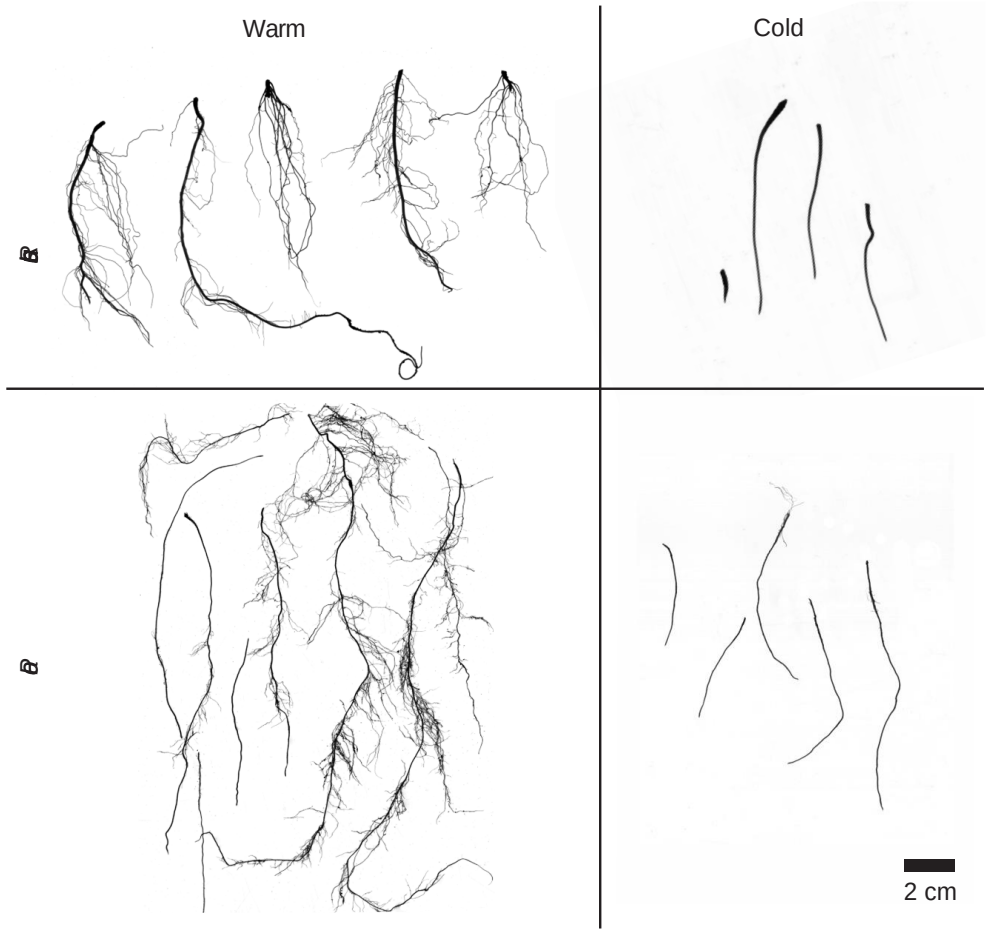
In contrast to the majority of root characteristics, aboveground plant parameters such as number of leaves (data not shown), leaf area, leaf mass and aboveground biomass were similar in warm and cold locations for a given species (Fig. 5.8, Tab. 5.2). Only Poa had significantly higher leaf mass and total aboveground biomass under the warm temperature regime, however, effects did not result in significant differences between locations (n.s. for posteriori Tukey HSD). LMF and SMF (including flowers) were also hardly affected by substrate temperature (although shorter pedicels were observed). SMF was only slightly lower in *Ranunculus* at the cold locations (by 23%, Tab. 5.2). And counter expectation, SLA was higher in cold treated *Ranunculus* by 20% ($P < 0.001$), in *Poa* by 16% ($P < 0.05$) and tended to be lower in *Homogyne* (Fig. 5.8).

Figure 5.6



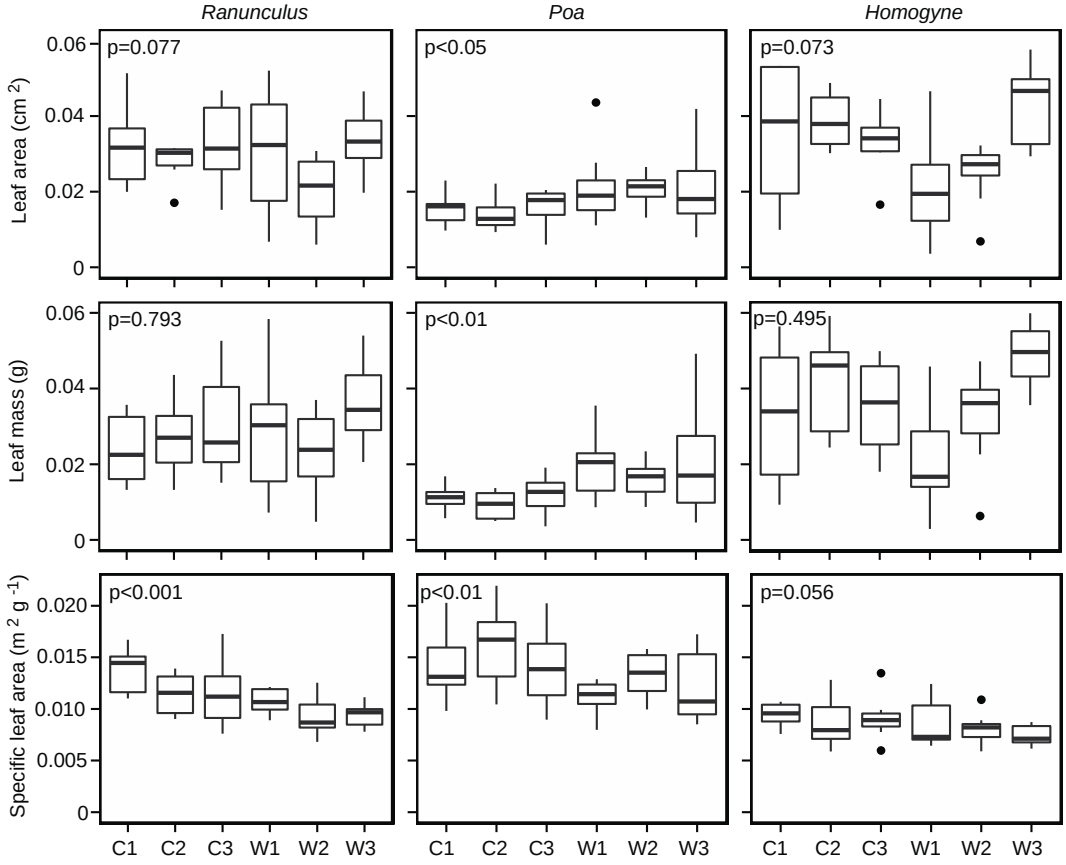
Root parameters at peak biomass for each location (n = 8 per location, boxplots and differences as explained in Fig. 4). Note the different y-axis scales and the missing values at C1 for *Homogyne* (no new roots).

Figure 5.7



Root systems of a plant individual under either cold or warm growing conditions (after 41 days). Note the difference in root length and branching.

Figure 5.8



Aboveground plant parameters at peak biomass for each location (n = 8 per location, boxplots and differences as explained in Fig. 4). P-values indicate significant difference between temperature regime (ANOVA). Note: Significant P-values indicate statistically significant difference between temperature regime, letters between locations (nested design, posteriori Tukey HSD).

Table 5.2: Above- (AGB), belowground biomass (BGB incl. rhizomes; both in g d.m.) and mass fractions (mean ± sd) of the three plant species at each location (RMF without rhizomes). P-values indicate significant difference between temperature regime and different letters between location (nested design, P <0.05). Note the missing value for BGB and RMF in *Homogyne* at location C1. C1-3: cold and W1-3: warm locations.

Species	Biomass/	C1	C2	C3	W1	W2	W3	P-value
Ranunculus	ABG	0.11 ± 0.04	0.14 ± 0.09	0.13 ± 0.05	0.16 ± 0.11	0.07 ± 0.03	0.12 ± 0.03	0.634
Poa		0.09 ± 0.03	0.09 ± 0.02	0.08 ± 0.03	0.13 ± 0.06	0.12 ± 0.06	0.13 ± 0.05	<0.05
Homogyne		0.16 ± 0.08	0.14 ± 0.04	0.15 ± 0.07	0.10 ± 0.05	0.11 ± 0.03	0.15 ± 0.04	0.197
Ranunculus	BGB	0.07 ± 0.01	0.09 ± 0.05	0.08 ± 0.02	0.13 ± 0.07	0.09 ± 0.04	0.14 ± 0.03	<0.01
Poa		0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.06 ± 0.02	0.07 ± 0.03	0.06 ± 0.02	<0.001
Homogyne		n.a.	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.101
Ranunculus	LMF	0.19 ± 0.04	0.21 ± 0.03	0.19 ± 0.05	0.14 ± 0.04	0.17 ± 0.04	0.17 ± 0.02	<0.001
Poa		0.18 ± 0.06	0.13 ± 0.06	0.19 ± 0.12	0.16 ± 0.08	0.16 ± 0.05	0.17 ± 0.11	0.902
Homogyne		0.32 ± 0.11	0.44 ± 0.11	0.39 ± 0.09	0.29 ± 0.16	0.33 ± 0.09	0.46 ± 0.12	0.574
Ranunculus	SMF	0.34 ± 0.09	0.28 ± 0.08	0.35 ± 0.07	0.32 ± 0.19	0.22 ± 0.06	0.21 ± 0.02	0.074
Poa		0.62 ± 0.11	0.62 ± 0.12	0.57 ± 0.14	0.58 ± 0.18	0.49 ± 0.12	0.51 ± 0.13	0.061
Homogyne		0.51 ± 0.13	0.38 ± 0.10	0.47 ± 0.06	0.49 ± 0.18	0.41 ± 0.09	0.34 ± 0.08	0.121
Ranunculus	RMF	0.38^{ac} ± 0.11	0.43^{a} ± 0.10	0.35^{a} ± 0.12	0.51^{bc} ± 0.15	0.57^{b} ± 0.09	0.59^{b} ± 0.03	<0.001
Poa		0.15^{ac} ± 0.11	0.16^{ac} ± 0.03	0.18^{a} ± 0.18	0.24^{b} ± 0.10	0.31^{b} ± 0.07	0.27^{bc} ± 0.06	<0.001
Homogyne		n.a.	0.01^{a} ± 0.01	0.02^{ac} ± 0.01	0.17^{b} ± 0.08	0.16^{b} ± 0.07	0.08^{bc} ± 0.03	<0.001

5.5 Discussion

We employed natural cold and warm substrate conditions to study the effect of low temperature on root and shoot development in three alpine plant species. The cold 'experimental water bath' provided by glacial and melt water streams allowed only few excursions to temperatures above 5 °C during periods of strong solar radiation, but predominately created substrate temperatures below 5 °C. Apart from the cold temperature, the selected plant species grew in neutral glacier silt enriched with a slow-release fertilizer (15 kg N ha^{-1} a^{-1}) and experienced otherwise 'normal' alpine summer conditions. Thus, cold substrate condition was the only manipulated and therefore limiting factor for root growth.

Roots clearly grew much less in terms of length and dry matter in cold compared to 7.9 K (mean across 41 days) warmer substrate, but aboveground plant growth (biomass) was hardly affected. Owing to the weather dependent variation in substrate temperature, regressions of root length increment *versus* concurrent temperature could be tested, which revealed close, linear positive correlations in the cold, but no such effect in the warm treatment, suggesting no further stimulation in root elongation at warmer temperatures by the 4-day resolution of these data. Only very little root growth was found when the mean temperature during census intervals was below 2 °C (Fig. 5.5), a threshold marginally lower than the one found for cold adapted evergreen and deciduous tree seedlings (Alvarez-Uria and Körner 2007, Schenker et al. 2014) but slightly higher than the close to 0° C threshold found earlier in leaves of *Poa alpina* (Körner and Woodward 1987) and in roots of arctic plants (Bliss 1956). Although, in the latter case, no continuous temperature records were available, hence, short warmer episodes may have remained unaccounted for. Part of the variation of mean root increment per census interval (Fig. 5.5) mirrors differences in root age, root position along the thermal gradient and individual plant vigour. At harvest, none of the cold treated roots of all three species reached the bottom of the pots where substrate temperatures approximated stream water temperatures of 1.5 to 2.5 °C. Longer-term temperature monitoring in glacial streams in the Furka region revealed monthly stream water temperatures of 1-2 °C for June and July (depending on onset of snowmelt), and 2-3 °C for August and September (person. comm. E. Hiltbrunner). Low temperatures also had a strong effect on root architecture. In our experiment, cold temperature inhibited the formation of second-order roots that are typical for alpine plants (Billings and Mooney 1968, Körner and Renhardt 1987). A pale,

almost white colour of cold treated roots and a low degree of branching were also reported for seedlings of European tree species when exposed to temperatures below ca. 7 °C (Schenker et al. 2014).

The duration of the experiment covered nearly the whole growing season at this elevation. However, the majority of roots of alpine plants commonly live several years. Based on radiocarbon signatures, Leifeld et al. (2015) reported a mean residence time of roots of about 15 years for an acidic grassland (dominated by the sedge *Carex curvula*). Thus, absence of branching in the cold treated roots could be partly explained by the young age of the observed roots. However, *Ranunculus glacialis* as a typical glacier forefield plant, behaves very differently from the sedge *C. curvula*. In *Ranunculus* every year new white roots emerge from the rhizome and last year's roots do not show a second expansion (Reisigl and Keller 1987). Hence, the size of the root systems achieved under experimental conditions can be considered final in *Ranunculus*. The situation may be similar to that in *Poa*, where each new shoot (tiller) within an individual builds its own adventitious root system. *Homogyne* certainly needs more than one season to build a fully developed root/rhizome system. In contrast to the other two species, *Homogyne* belongs to late succession alpine grassland communities that experience a growing season longer as we provided here in the glacier forefield.

The *in situ* fine root length per unit leaf area in 25 species grown at >3000 m elevation was much greater than in congeneric plants grown at 600 m, reflecting the long-term net outcome of new root growth and root mortality (Körner and Renhardt 1987). Low temperatures may not only limit root growth, they may also delay aging in roots 'designed' for multi-year functioning, hence, higher root longevity is a most likely explanation for the differences in fine root length in mature root systems (see Leifeld et al. 2015). Both, *Ranunculus glacialis* and *Poa alpina* are pioneer species on often unstable ground, a possible explanation of their lower root longevity. In the study of Alvarez-Uria and Körner (2007) low temperatures induced an overall reduction in SRL in cold adapted tree seedlings, irrespective of the species. In the present study, a pronounced decrease in SRL was noticed in *Poa* and to lesser extent in *Ranuculus*. The thick, hardly branched roots of *Ranunculus* are also typical for plants in wet habitats.
Despite our moderate fertilizer addition, it remains open whether plants were able to take up these nutrients under cold conditions. However, cold conditions were not exceptionally low for these plants, and aboveground biomass formation did not show any signs of nutrient deficiency.

A plausible explanation for the similar number of leaves, leaf area and the small differences in SLA between temperature treatments (statistically significant in *Ranunculus* and *Poa*) might be the known pre-formation of advanced leaf and shoot primordia in the previous season (Körner 2003). Further, all plant individuals in this experiment were exposed to similar aboveground temperatures, and presumably, the cold conditions near the soil surface did not affect differentiation of leaves and stems. In both, *Poa* and *Ranunculus,* the apical meristems were a few millimeters, up to one centimeter, below the soil surface, and thus, were experiencing at least part of the cold treatment, which might have caused the delayed flowering and the development of shorter stems and smaller flowers (SMF), in comparison to the warm (*sensu* normal) temperature treatment.

Our data do not permit to define an absolute minimum temperature for root expansion, because we measured root elongation rates over 4-day intervals only, including day-night cycles of temperature and varying weather conditions. However, we can relate the thermal sums calculated from hourly temperature readings to the census data. We thus, can identify a minimum of degree hours (°h) required for roots to grow. The smallest mean root elongation within four days (5.2 mm for *Ranunculus* and 6.0 mm for *Poa*, Fig. 5.5) was measured in the coldest census interval at the location C3 (*Ranunculus*) and C1 (*Poa*), respectively, where temperatures did hardly exceed 2 °C within the four days, yielding thermal sums of 19 °h (*Ranunculus*) and 13 °h (*Poa*) >2 °C and 1 °h and 12 °h >3 °C. We assume that the threshold for minimum root growth of the two species is close to 2 °C. Root growth under such temperature conditions corresponds to a minimum root elongation of roughly 1-1.5 mm per day according to our data, which refers to 26% of the mean root elongation of both species under warm conditions.

The data obtained here suggest that the bulk of new roots was produced at temperatures above 5 °C, with the length increment approaching zero when temperatures drop below 2 °C. Both *Ranunculus* and *Poa*,thus, make no exception to the general pattern of temperature responses of tissue formation as was previously observed in trees and winter crops. However, the definition of an absolute lower temperature limit for root growth is of limited value in the case of an obviously asymptotic process. Provided long enough periods, some root extension may even occur at 0 °C as was reported for roots in the Caucasian snow-bed plant *Corydalis conorhiza*. These plants actually develop thin networks of fine snow roots into snow pack during winter (Onipchenko et al. 2014). Given the temperature sensitivity of root growth, very brief daytime excursions to warmer temperatures under the influence of direct solar irradiance are sufficient for root development under otherwise cold life conditions. As soon as temperatures approach 10

°C, there is no further gain in root growth. Hence, there is a narrow thermal window that permits root system formation with no advantage if temperatures get warmer.

Maximum root elongation rates under our cold soil conditions reached the level of the minimum rates measured under warm conditions (overlapping data between cold and warm locations in Fig. 5.4, Fig. 5.5). Rates between 12 and 15 mm per 4-day census interval (corresponding to ca. 40 °h >5 °C or ca. 10 °h >5 °C per day) appear to be necessary to achieve a fully developed root system within a short alpine summer. We conclude that the studied alpine species grow well at soil temperatures between 5 and 10 °C, irrespective of aboveground temperatures. The absolute minimum temperature for growth in our study plants is close to 2 °C, in contrast to the thermal requirements for carbon acquisition (photosynthesis), which reaches at least 30 % of maximum rates at such temperatures (Körner 2003), underpinning the critical role of sink activity (growth) under low temperature.

5.6 Acknowledgements

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5.7 References of additional Chapter 5

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6 Summary and conclusions

Temperature is a major driver of plant growth in cold ecosystems such as boreal, arctic and alpine environments and of winter crops in temperate regions during winter and springtime. Plant growth and productivity in those settings are presumably constrained by low temperature through slow tissue formation (sink activity) rather than photosynthesis (source activity). Limiting temperatures occur seasonally and thus define the duration of the growing season and/or set distribution limits of plants. Moreover, the occurrence of limiting temperatures under naturally fluctuating temperatures can also result in diel (during 24 h) variations of the growth rate of leaves or roots. Yet, the exact temperature thresholds and the growth dynamics under critically cold conditions are not well understood. Such data are needed to explain the success of wild plants and winter crops under critically low temperatures.

Plant growth is defined as the irreversible volumetric and mass increase of plant tissue. For practical reasons growth dynamics (elongation or expansion of organs) are commonly monitored non-destructively by length or areal expansion records, but these must be tuned with mass increment. Low temperature limits all physiological processes involved in plant growth at the cell and tissue level. However, the processes that might represent a thermal bottleneck for growth are still unclear. It is quite evident that low temperatures hardly affect photosynthetic carbon capture. Thus, it has been suggested that processes related to cell wall synthesis, particularly the organisation of microfilaments and microotubuli of the cytoskeleton are sensitive to, and can be impaired by low temperatures. Further, growth-regulating factors that control development and intrinsic growth rhythms, such as the circadian clock can become disrupted at low temperatures. Since functional leaves and roots also require a lignified xylem, xylem lignification may be another constraint.

To elucidate the thermal limits for growth of leaves and roots, accurate, non-destructive and particularly continuous measurements with short measurement intervals are essential. Modern image sequence analysis methodologies offer the possibility to collect growth data of single leaves or roots with high spatial and temporal resolution and a considerable sample size. To test and further develop these techniques for an open-air field application was one of the methodological challenges of the present thesis.

The overall hypothesis of this doctoral thesis was that all cold adapted plants, regardless of whether they are alpine species or winter crop varieties are limited by similar temperature related limitations and are constrained by similar thermal thresholds. This

hypothesis was tested by three field experiments exploring the thermal limits of leaf growth in monocot (Summary figure 1A, Chapter 2) and dicot winter crops (Summary figure 1C, Chapter 3) and for root growth in alpine plants (Summary figure 1E, Chapter 4). Both, leaves and roots are expected to be affected by similar temperature related limitations at the cell and tissue level.

6.1 Methods

In a first experiment, rates of leaf elongation in graminoid crops including wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) were studied during cold springtime conditions. A second experiment put a spotlight on leaf expansion of rapeseed (*Brassica napus*) during winter. Both studies used natural fluctuating temperatures in the field between -5 and + 20 °C as a low temperature treatment and were conducted at the ETH research station for plant sciences Lindau-Eschikon. Leaf growth in both, monocots and dicots was measured based on a high-resolution marker tracking image sequence analysis approach, for which an appropriate mechanical setup was developed during this thesis. The movements of white beads, connected to the margins of growing leaves were used as artificial markers to track leaf growth in terms of leaf elongation rates (monocots, Summary figure 1A, Chapter 1) or relative areal expansion rates (dicots, Summary figure 1C; Chapter 2). In the third experiment, root growth in the following alpine plant species was investigated: one grass *Poa alpina* and three herb species *Ranunculus glacialis, Rumex alpinus* and *Tussilago farfara*. Plants were grown outdoors at the alpine research station ALPFOR in 2440 m in the Swiss central Alps with a tightly controlled and monitored thermal root environment (Summary figure 1E, Chapter 3). Roots grew against a steep vertical soil temperature gradient, so that the low temperature limit of root growth could be identified. During the experiment, root growth rates were obtained from roots growing along the transparent cylinder walls based on image sequence analysis and were combined with soil temperature data. At harvest, final rooting depth and the corresponding limiting low temperatures were determined. Tissue from root tips that arrived at the physiological low temperature 'barrier' was sampled and examined for histological traits. Thin longitudinal sections were analyzed for the cold temperature effect on cell elongation/differentiation and xylem lignification.

6.2 Results

Leaf length tracker: a novel approach to analyse leaf elongation close to the thermal limit of growth in the field[1]

The developed and employed method has proven to be suited for measuring leaf elongation in cereals and other graminoids under field and controlled conditions. The obtained leaf elongation rates were precise enough for both, to determine the low temperature limit for leaf growth in monocots and to quantify subtle genotype-specific responses of growth and temperature. Leaf elongation in the studied winter cereals reached zero rates at 0 °C air temperature measured at 5 cm plant height (Summary figure 1B). Leaf growth rates were highly correlated with air temperature at plant height and significantly increased above 5 °C. The diel leaf growth dynamic was mainly affected by fluctuating air temperatures around leaves (Summary figure 1B). Leaf growth in the studied wheat, barley and ryegrass varieties showed a genotype specific response to temperature. In wheat and barley, some varieties grew more vigorously at lower temperatures (>5 °C), others showed enhanced growth at higher temperatures (<5 °C) and some varieties had a strict linear growth relation with temperature and could not profit from higher temperatures.

[1] Nagelmüller S, Kirchgessner N, Yates S, Hiltpold M, Walter A. 2016. Leaf length tracker: a novel approach to analyse leaf elongation close to the thermal limit of growth in the field. *Journal of Experimental Botany* 67: 1897-1906.

Diel leaf growth of rapeseed at critically low temperature under winter field conditions[2]

Similar to the winter cereals, leaf growth in the dicot rapeseed plants stopped at 0 °C air temperature (5 cm plant height). However, the response of leaf growth and temperature is more complex and rates of leaf expansion are co-influenced by vapor pressure deficit and daytime (circadian rhythm, Summary figure 1D). Growth rates at noon or early afternoon showed a pronounced depression, which was related to the higher evaporative demand during peak vapor pressure deficit. Thus, leaves of the dicot rapeseed showed pronounced diel growth patterns, with peak leaf expansion during the night despite lower temperatures. Such diel patterns were consistently observed above an air temperature threshold of 4 °C. These diel leaf growth variations above 4 °C were described by a regression model based on leaf age and the environmental factors vapor pressure deficit (VPD), temperature and light with VPD having the strongest negative effect on leaf growth (Summary figure 1D). Regular 24 h fluctuations of the model residuals over time and growth peaks at night that were not covered by a model predicted growth rate seem to reflect an endogenous circadian rhythm component controlling dicot leaf growth above 4 °C.

Low temperature limits for root growth in alpine species are set by cell differentiation[3]

The experiment with alpine plants confirmed that root growth below 5 °C is strongly inhibited compared to a 10 °C control group, which resulted in a poorly developed root system with hardly any root branching. Absolute minimum temperatures for root length increment in these cold-adapted species were found between 0.8 and 1.4 °C. These zero points of growth are associated with characteristic changes in the apical tissues' structure. Meristematic root cells were inhibited to expand beyond a certain temperature limit and led to a shortened root elongation zone (Summary figure 1F). The xylem in these apical tissues was less lignified, however, proper lignification is essential to provide water-tight and functional vessels. These findings suggest that cell differentiation and xylem lignification are decisive

[2] Nagelmüller S, Yates S, Walter A. 2017. Diel leaf growth of rapeseed at critically low temperature under winter field conditions. Submitted to *Functional Plant Biology*

[3] Nagelmüller S, Hiltbrunner E, Körner C. 2017. Low temperature limits for root growth in alpine species are set by cell differentiation. *AoB PLANTS* 9:plx054. doi:10.1093/aobpla/plx054

processes that feed back on cell production and prevent any further extension of root tips into even colder soil space.

6.3 Discussion and conclusions

This thesis focused on continued plant growth measurements in the field to examine *in situ* plant growth constraints at low temperature. Such data were underrepresented in the literature so far and measurements with the here achieved temporal and spatial resolution are novel for field experiments. The thesis also underlines the significance of precise recordings of the thermal environment for plant growth analysis, which should be obtained as close as possible to the studied leaves or roots. Longer distances between the growing plants and the temperature sensor, even an air temperature sensor at 2 m height cannot depict the exact temperature fluctuations around the leaves. Moreover, daily or lager mean temperatures can be misleading because they may mask periods with higher temperatures that facilitates growth to have happened. Both, inaccurate sensor positions and averaged temperatures therefore lead to false conclusions regarding relationships of growth and temperature.

It is concluded that the low temperature limit for leaf growth in cold adapted plants, the so-called base temperature, is at 0 °C and is similar for both, monocot and dicot plants. However, differences were observed with respect to the growth dynamics with rising temperature and the influences of other environmental parameters. While the leaf growth dynamics in monocots are strictly temperature controlled, dicot leaf growth at low temperatures shows a transition from pronounced environmental regulation below 4 °C and a superposition of environmental and internal, circadian-clock-dependent regulation.
The absolute thermal limit for root growth (1-2 °C) was slightly higher compared to that of leaves (0-1 °C), which may be related to the fact that roots grow in a more buffered environment, while the temperature variation in leaves can still allow some brief warm up periods, not captured by air temperature. It may also be that expansion continued towards 0 °C (water influx) while growth actually did not. These findings support the initial hypothesis that the limitation of plant growth by low temperature is similar in all cold adapted plants. Thus, the adaptation to low temperature is a uniform evolutionary mechanism, responsible for the success of arctic alpine plants or crops in winter.

The temperature conditions were never constant and naturally fluctuated during the day in all experiments. Even a controlled treatment in the field does not permit to freeze

temperatures when ambient conditions vary. Without such small thermal variations growth of leaves and roots but might have inhibited completely. Therefore, the concept of threshold temperatures can help to overcome such difficulties in field experiments. Evidence for a critical role of temperatures between 4 and 5 °C for the bulk of roots or leaf formation was found in all three studies. Leaf growth in winter wheat was enhanced above such a threshold, diel regulation of leaf expansion in dicot rapeseed leaves were only detected above 4 °C and the majority of root biomass production in alpine plants was produced above 5 °C.

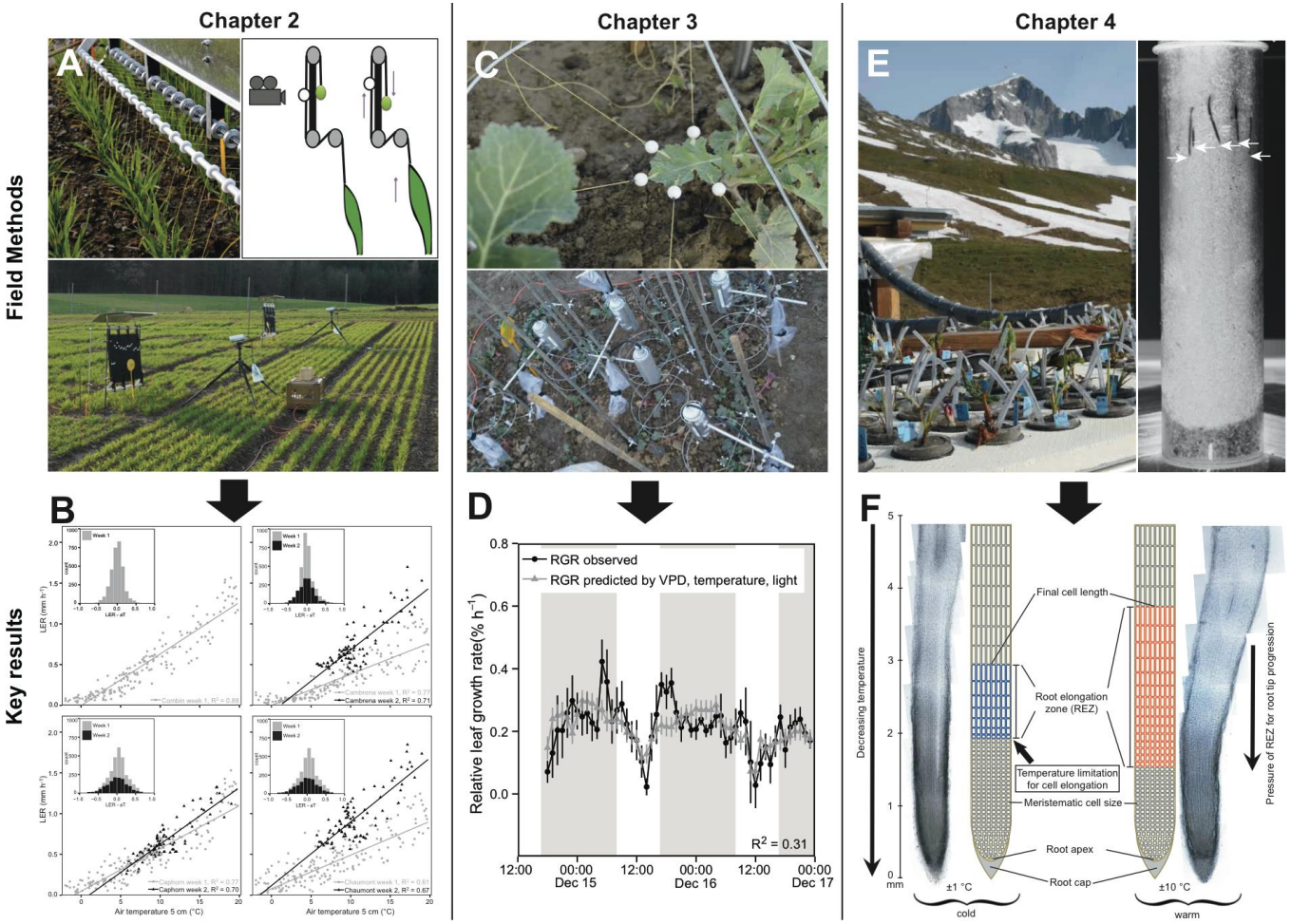
The detected genotype specific responses of cereal leaf growth/elongation to temperature appear to be promising for breeding for cold tolerant winter crops that have the potential to accumulate more biomass during winter. Such varieties could be harvested earlier in the season to avoid summer droughts and thus increase yield stability in the context of climate change.

According to the literature, cell division should have still been active in the leaf and root meristems close to the minimum temperatures between 0 and 1.4 °C. However, due to the steeply decreasing soil temperature gradient, cell expansion might have become impaired already above these minimum root tip temperatures and terminated root growth. These thermal limitations of cell expansion are awaiting a biochemical clarification but are presumably related to limited cell wall synthesis. Similar physiological limitations might also apply to leaf growth but this remains to be proven by histological examinations of cold grown leaf tissues. Yet, for such studies to make sense, such leaves must not enter warm up excursions during the day. Natural temperature fluctuations might have enabled cell wall synthesis by shifting these processes to warmer hours at daytime.

A further limiting mechanism that can contribute to reduced plant growth in the cold are possibly disrupted endogenous rhythms at low temperature in dicot species. Transcriptome studies could help to investigate such developmental (cell differentiation) or clock related thermal limitations in more detail.

This thesis presents developmental and cellular explanations of plant growth in the cold. The results are both useful to explain and define the growing season and the distribution limits of arctic-alpine plants, and to assess the performance of cold tolerant winter crops for plant breeding. The methods refined during this thesis enable *in situ* measurements under natural field conditions as well as in crop fields, and they will permit advances in our understanding of other growth related processes under field conditions and thus present a promising tool for scientist and breeders.

6.4 Summary figure



The upper row shows the experimental field situation and the applied methodology to measure leaf growth in winter cereals (Chapter 2), in dicot rapeseed (Chapter 3) and root growth in alpine plants (Chapter 4). The lower row highlights some key results. More details can be found in the chapters of this thesis.

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8 Supplementary material

8.1 Supplementary material to Chapter 2

Figure S2.1: Manual for Leaf Length Tracker (LLT) version 1.01

The software is made for tracking artificial landmarks (beads) and calculating leaf elongation rates using an image sequence of the leaf length measurement panel. It is free for download at https://sourceforge.net/projects/leaf-length-tracker/.

It is written in Matlab and compiled for standalone usage in Version 2013b 64 Bit on Windows. For running it, MS Excel and the Matlab MCR 2013b (available for free at http://ch.mathworks.com/products/compiler/mcr/) must be installed on the computer. We recommend to use a computer with enough RAM to process the data (we used an Intel® CoreTM i5 processor and 4 GB RAM).

The provided test data at Sourceforge is a short and reduced image sequence compared to the original data used in the article and therefore only useful for a fast general check of the principles of the software and the requested file structure.

Requirements:

An image sequence of your measurement panel with a time interval of your choice and a calibration image of a checkerboard (we used a square size of 45.5 mm) of the size of your panel. Markers must move in y-direction.

Each image in the sequence needs to be named in the following format:

20141119_CH01_164414s.jpg

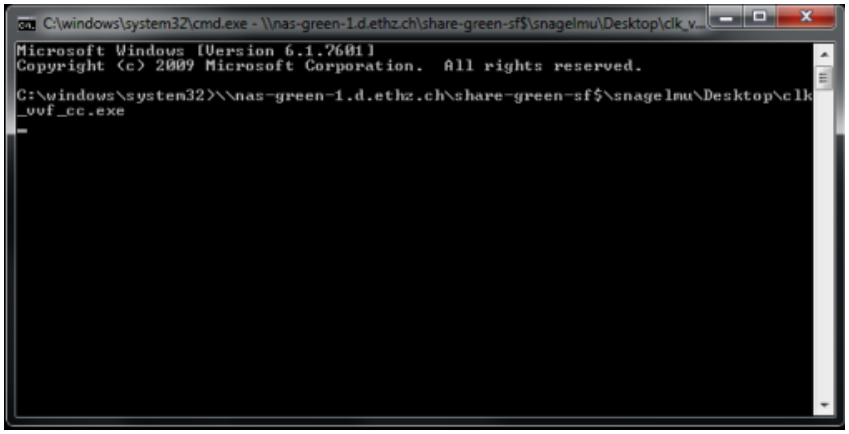
where the first number is the date information (yyyy,mm,dd). The second term CH01 is a name code for your image sequence (choose CH01-CH99). And the last number the time information (hh,mm,ss). The recommended image resolution is at least 2 pixel mm^{-1}.

If you are using a LupusNET HD camera (LUPUS-Electronics, Landau, Germany), you can use the program “Invid_lupus_read” also contained in the zip-file to rename your images. You can also use the program “Bulk Rename” (http://www.bulkrenameutility.co.uk/Download.php) to name your images accordingly.

All images need to be in the same folder, which should also contain another subfolder named “Calibration”, containing the checkerboard calibration image.

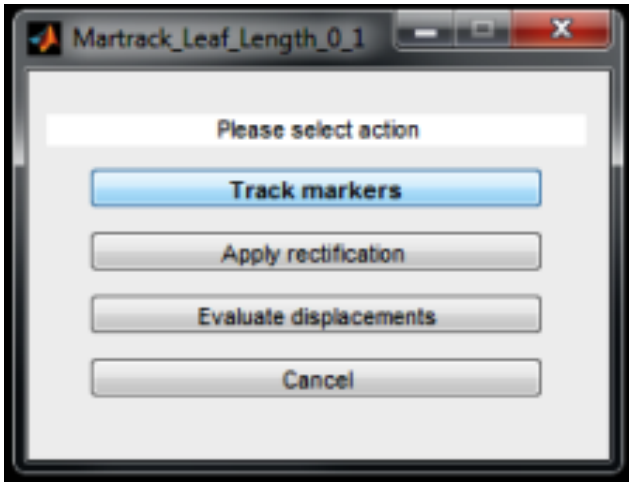
Now you are ready to start the analysis following the instructions.

1. Open a Dos window by opening start menue, searching for “cmd” and executing cmd.exe. Drag and drop Leaf_lenght_tracker.exe into the shell window, activate it by mouse click, and press “enter”.

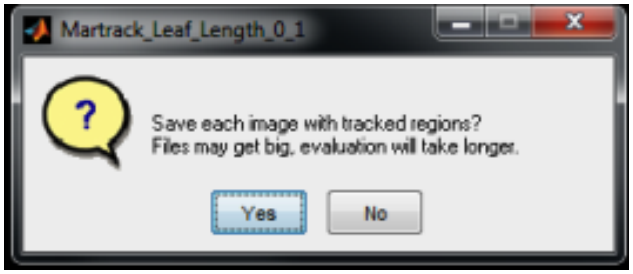


2. If you are using “Leaf Length Tracker” for the first time, the following license agreement opens. Klick “I read the complete above license agreement. I understand and agree” to proceed.

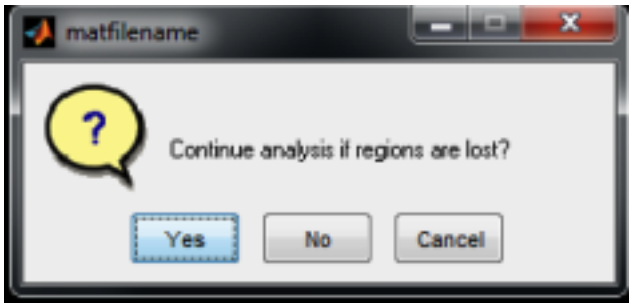
3. The software interface opens up and provides the single analysis steps in the recommended order: Track mackers, Rectification (Correction for lens distortion) and calculation of displacements. You can also choose the programms individually once the previous analysis has been done before. Choose “Track makers” to start the tracking procedure.



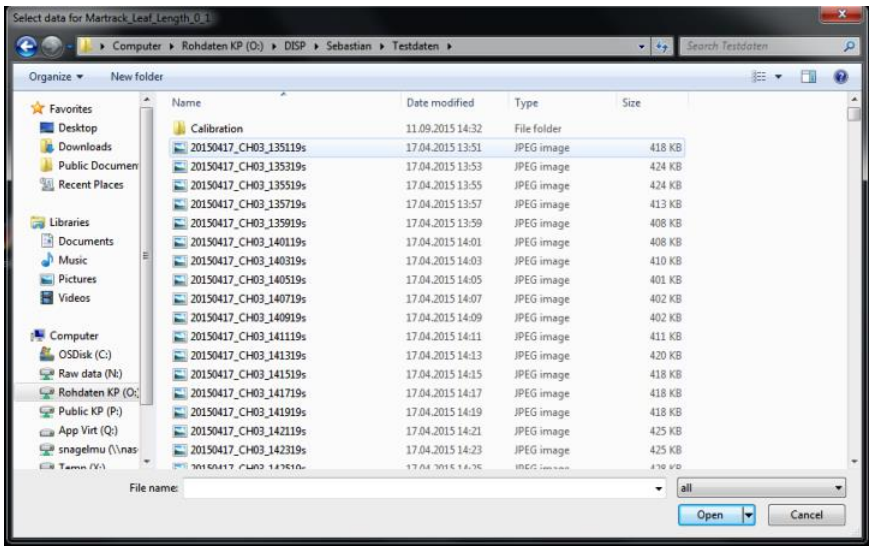
4. A new window opens and asks, if you want to save each image with the tracked regions. Those images show the tracked bead position and are saved as a TIFF stack. (If you chose yes, the evaluation will take noticeably longer.)



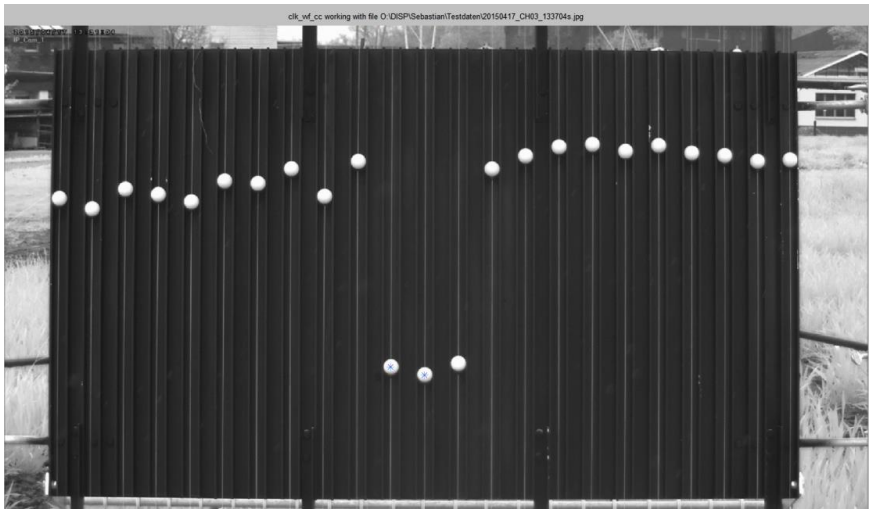
5. The next window asks if you want the programm to contiue the analysis if a region (bead) is lost. If answering “yes”, the software tries to localize the bead in the next image of the sequence and deletes the displacement data of the particular bead in the problematic image. When clicking “no”, the program stops the tracking of the particular bead.



6. Browse to your image folder and select the first image of your sequence

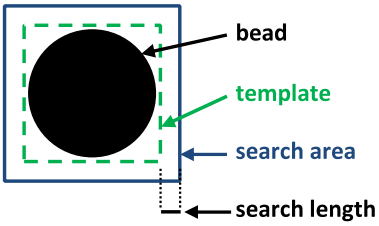


7. The program opens the first image were the markers need to be selected by mous-klicking into the center of the beads. Backspaces remove the last marked position. Double-clicks or „enter“ finish the bead selection. We recommend to select the three reference beads first (lower three beads in image below).

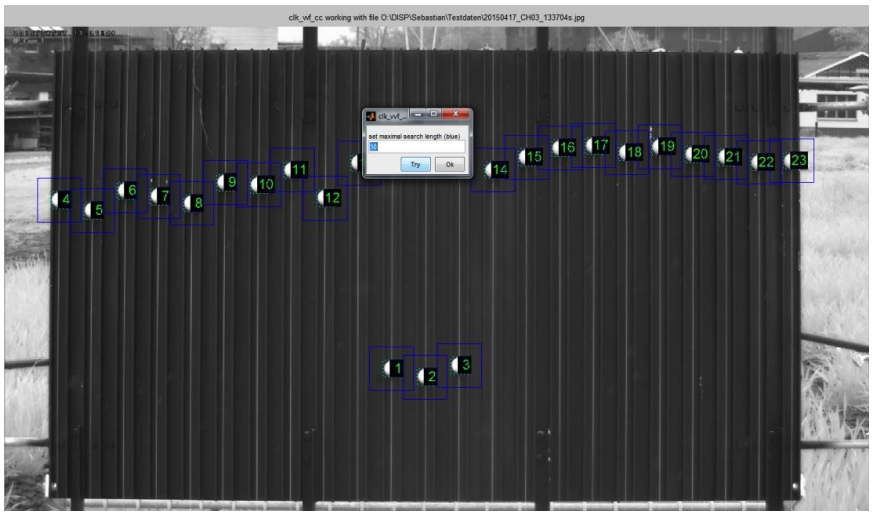


8. Now you are asked to set the template size around the beads. Typ a new template size, and click „Try“ to visualize as image overlay. As soon as the template size fits well arround the beads, click “Ok” to continue.

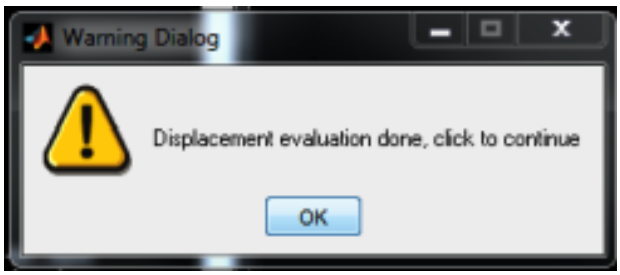
9. As a last step you have to define the search length around the beads. Type a new search length, and visualize the corresponding search area as image overlay by clicking “Try”. We recommend a seach area size between 30 and 40 pixel. Click “Ok” to continue, which starts the tracking.



© Mielewczik et al. (2013)

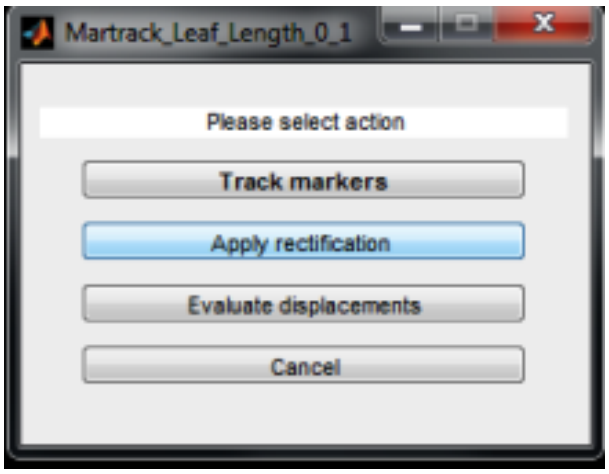


10. Now the calculation starts, and every bead is tracked throughout the whole sequence. The following window opens when the tracking is finished.

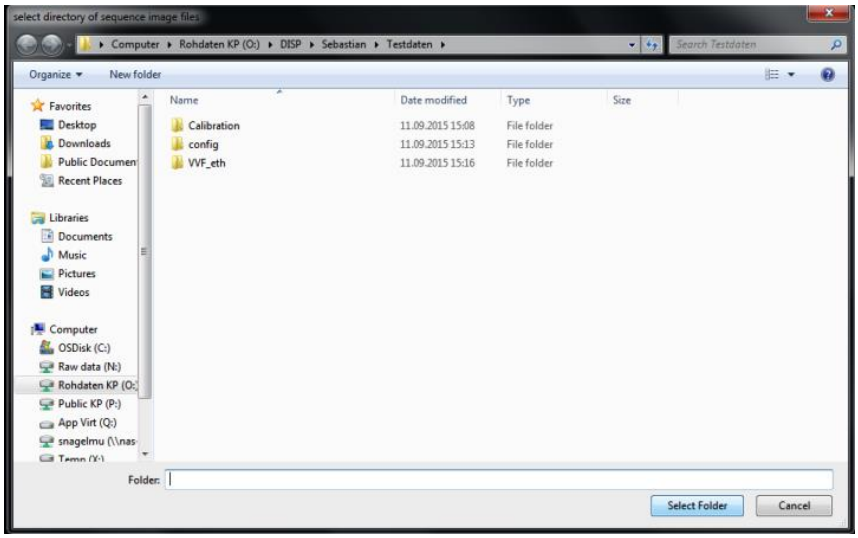


After clicking “Ok” figures with the crosscorrelation coefficient of regions over the image sequence and the number of found regions over the frame number are displayed. The tracking dispalcements are stored in the result subfolder “VVF_eth”, which will be used for calculating leaf length displacements in milimeters.

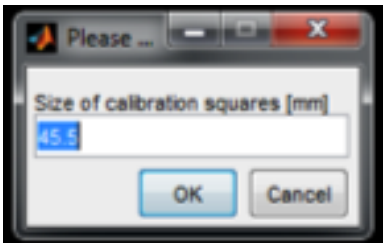
11. The second step in the menue is the “Apply rectification” function, wich corrects your images for lens distortion and automtically converts the pixel coordinates into milimeters.



12. Browse to your folder containing the “Calibration” folder and choose “Select Folder”.

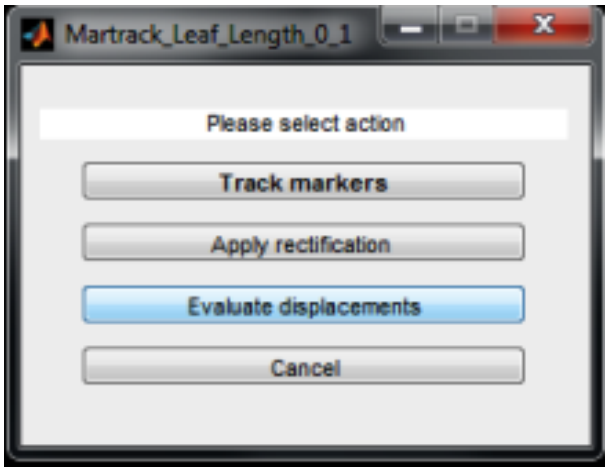


13. A window opens and ask for the size of the calibration squares. Typ the square size in milimeters and click “OK”.

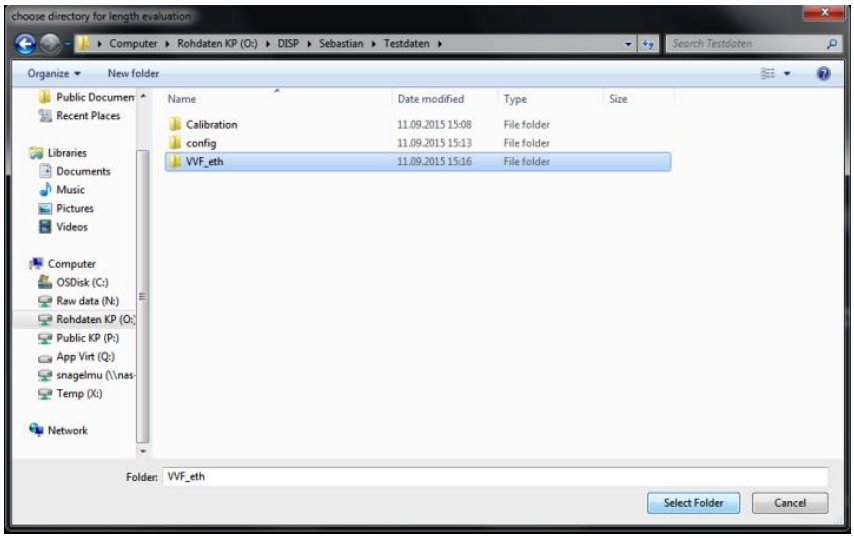


14. Images of the checkerbord and the first image in the sequence are displayed in the “original” and “corrected” version and stored in the subfolder “Check” in the “Calibration” folder.

15. To finish the analysis, select “Evaluate displacements” in the user interface.



16. Again, browse to your image folder and mouse-clike on the folder “VVF-eth” and click select folder.

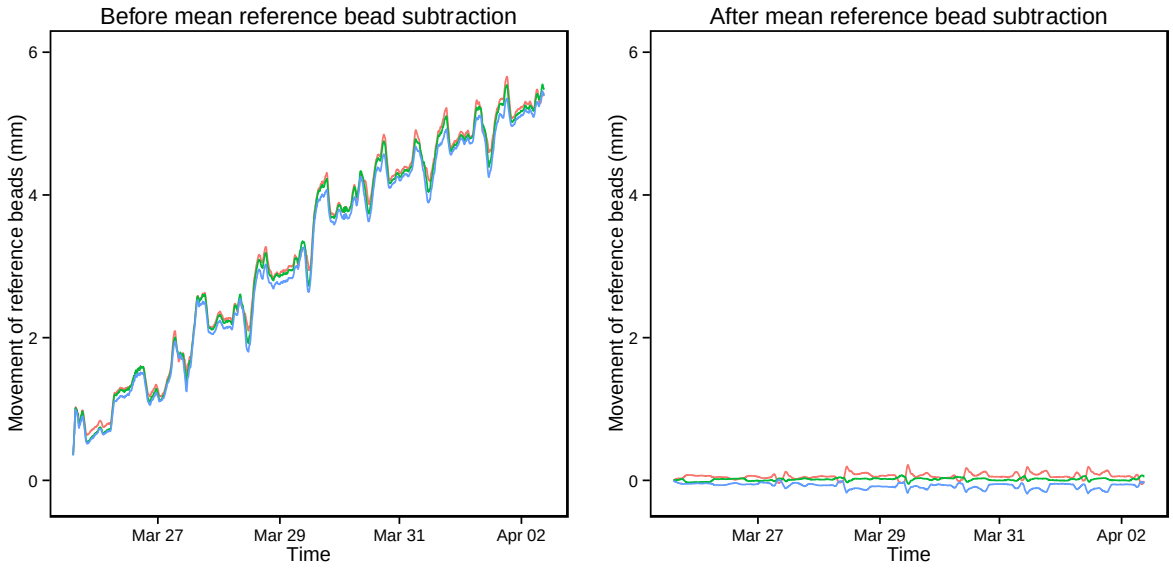


17. A window informs you that data, corrected for lense distortion is used. You can close it by clicking ok.

18. Exit the software by clicking “Cancel” in the user interface

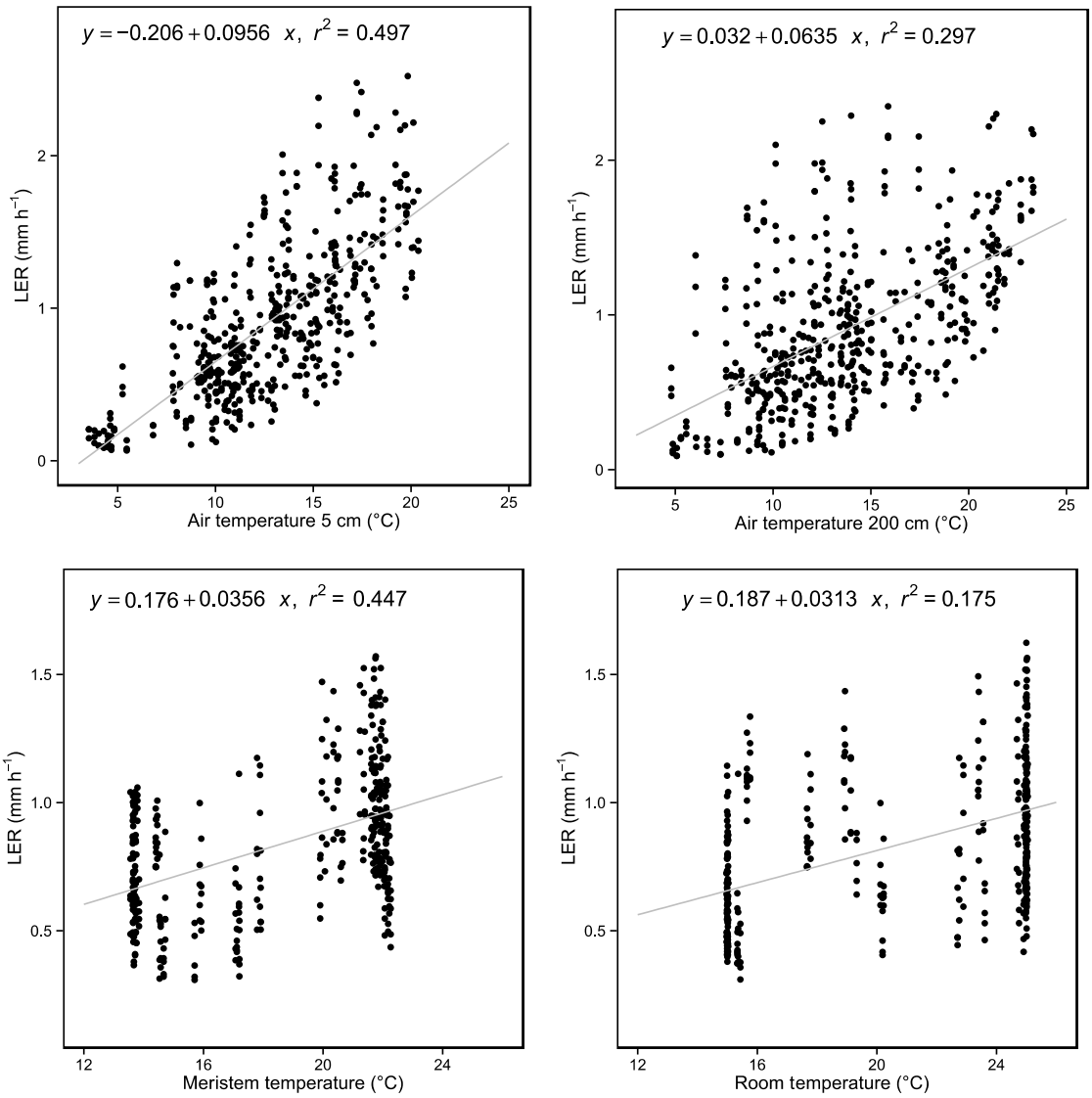
When calculations are finished figures with displacement (mm) and LER (mm h^{-1}) over time are displayed. In the subfolder “Length_sph1_corrected” in the result folder “VVF_eth” you can find different figures and a Microsoft excel-file containing an extra sheet for each tracked bead Lx – Ly (with columns “displ (mm)” for displacement e.g. leaf length, “displ (mm) smoothed”, “d displ smoothed / d time (mm/h)” e.g. leaf elongation rate and the “date” in the format dd.mm.yyyy hh:mm).
Now you can start data analysis and work with the leaf length data (displ) or elongation rates (d displ smoothed / d time (mm/h)). The elongation rates presented in the article were calculated with the R Statistical Software (R Core Team 2014) using the “displ (mm)” and “date” columns. We therefore recommend R for data analysis and the package “gdata” (Warnes et al. 2014) to read in the result excel-file.

Figure S2.2



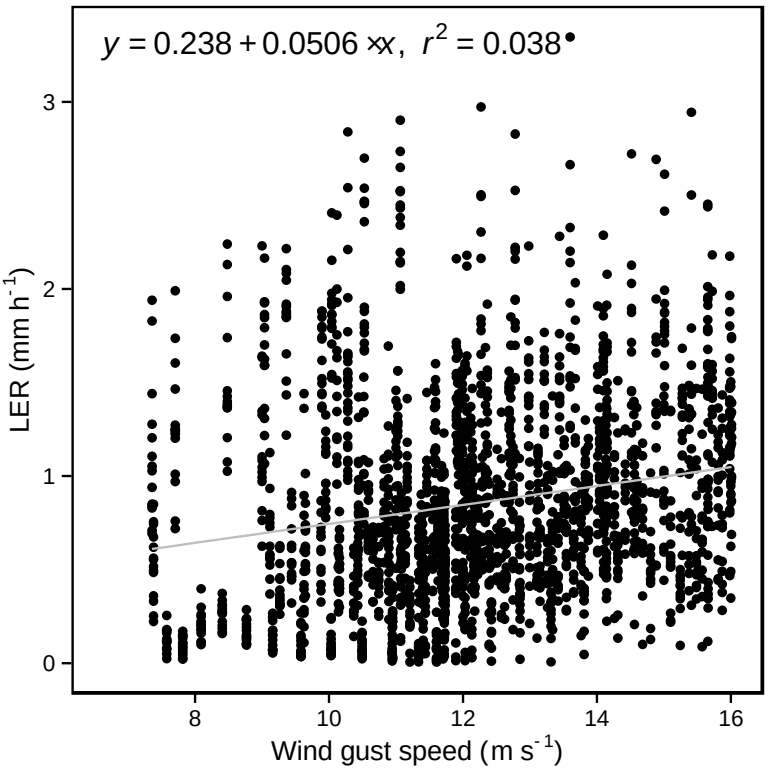
Left: Example for the movement of three reference beads over time (taken from the wheat measurement in week 1) that were not attached to leaves. Short-term movements are due to wind and moisture induced thread stretching, the long-term displacement can be explained by small position shifts of the camera tripod relative to the measurement panel. Right: The result when subtracting the mean reference bead displacement from reference beads itself, which improved the accuracy of our measurements.

Figure S2.3



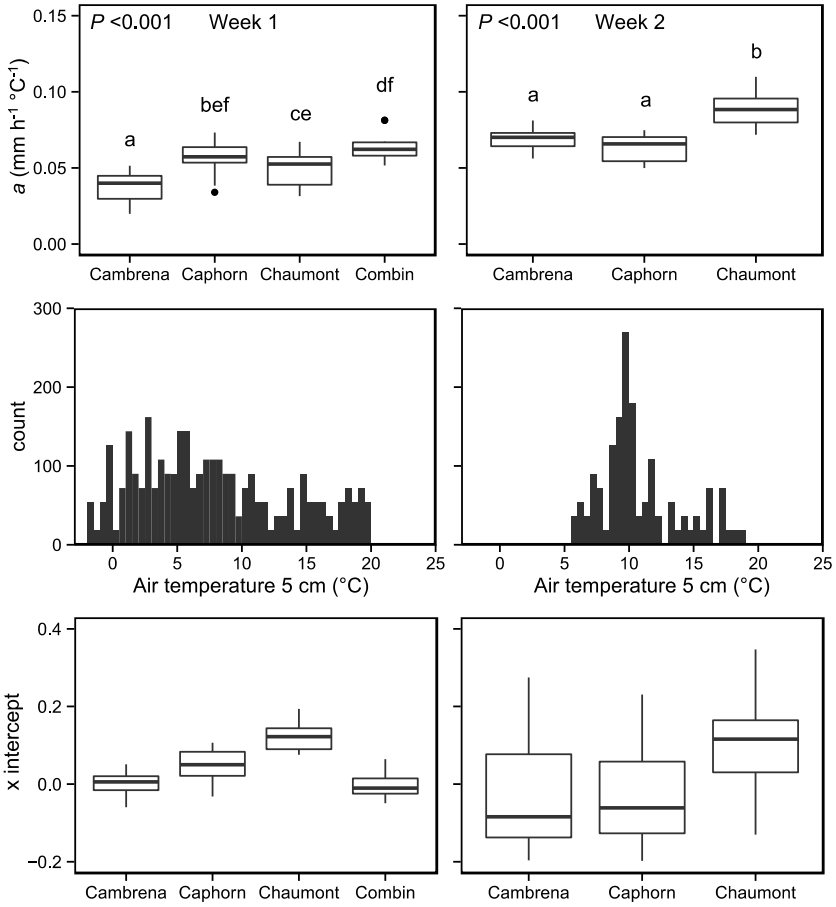
Upper row: Correlation of mean LER of summer barley (example from the Kunkels study site) to temperature at plant height (air temperature 5 cm) and in 200 cm height. Note the higher R^2 values for temperature data in 5 cm and the slightly different temperature range. This was consisted in all field measurements. Lower Row: Correlation of mean LER of ryegrass using meristem temperature (left) and room temperature of the climate chamber. Again, note the higher R^2 values for meristem temperature data and the slightly different temperature range.

Figure S2.4



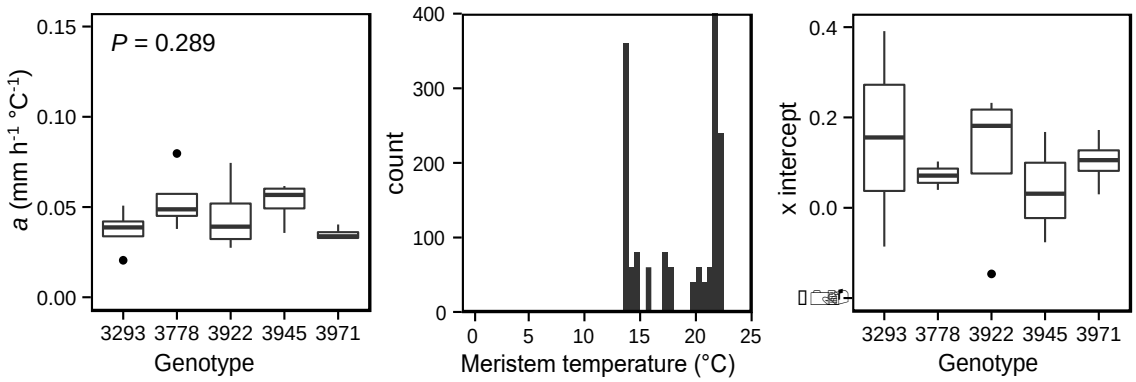
Correlation of LER of summer barley against wind gust speed from the most windy weather period at the mountain Kunkels study site. There is no correlation with wind gusts of up to 16 m s^{-1}.

Figure S2.5



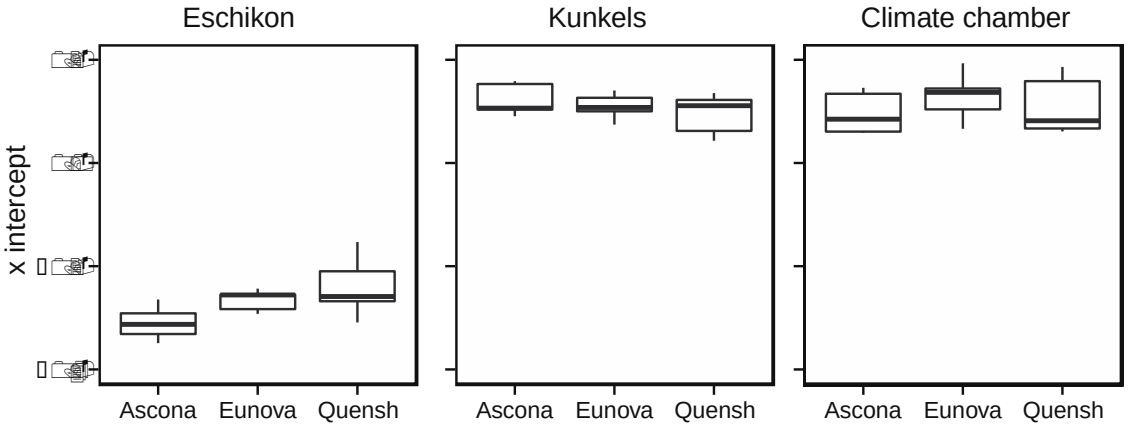
Upper row: LER per °C (*a*) of four winter wheat varieties (each n = 20 leaves) from week 1 (left) and week 2 (right). *P*-values are derived from ANOVA and letters above boxes indicate significant genotype-specific differences (Tukey-HSD, P <0.05). Middle row: Histograms of mean temperature per hour (in steps of 0.5 °K) from the period of measurements. Lower row: X intercepts close to 0 °C for all wheat genotypes (derived from normal linear correlations of LER and temperature without fitting trough zero). This did slightly change the ANOVA *P*-values but did not change the significance or Tukey-HSD results.

Figure S2.6



Left: LER per °C (a) of five ryegrass genotypes (each n = 4 leaves). *P*-value is derived from ANOVA. Middle: Histograms of meristem temperature per hour (in steps of 0.5 °K) from the period of measurements. Right: X intercepts close to 0 °C for all genotypes (derived from normal linear correlations of LER and temperature without fitting trough zero).

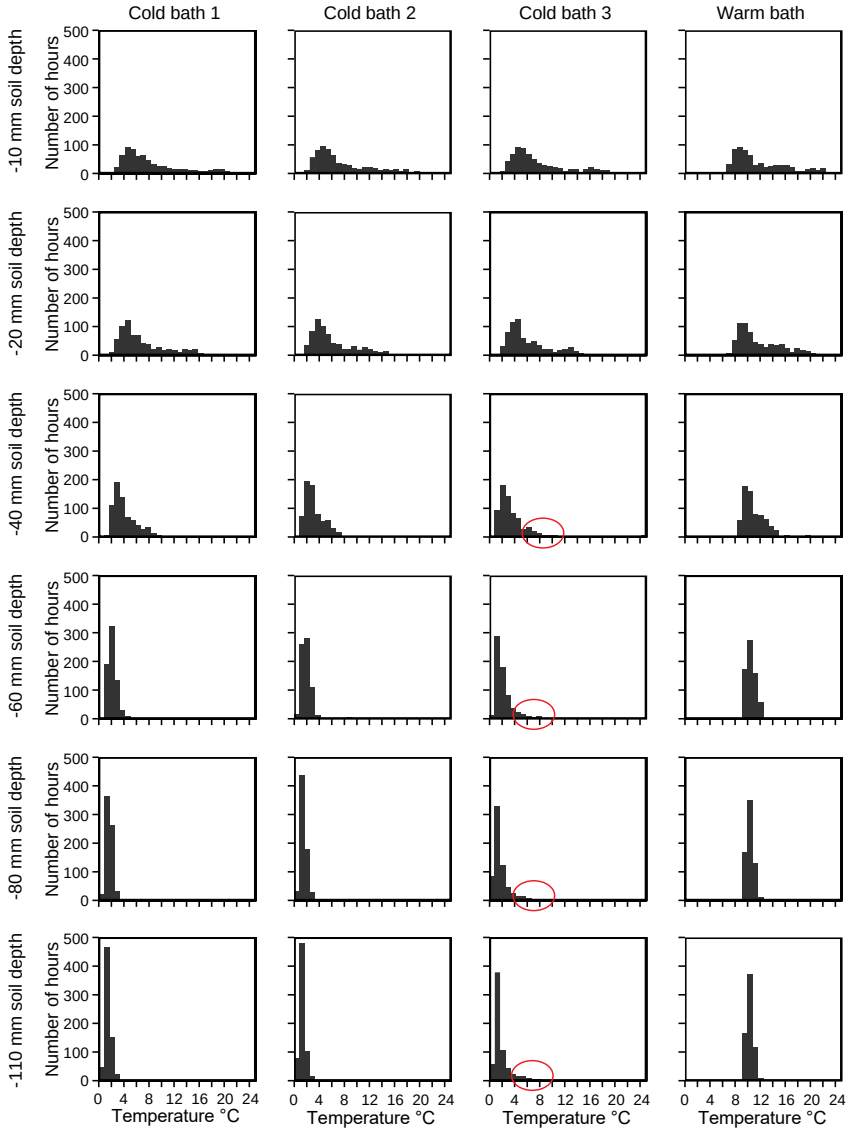
Figure S2.7



X intercepts close to 0 °C for all three summer barley genotypes at the two field sites and in the climate chamber (derived from normal linear correlations of LER and temperature without fitting trough zero). Again, this did slightly change the ANOVA P-values presented in the article but did not change the significance or Tukey-HSD results.

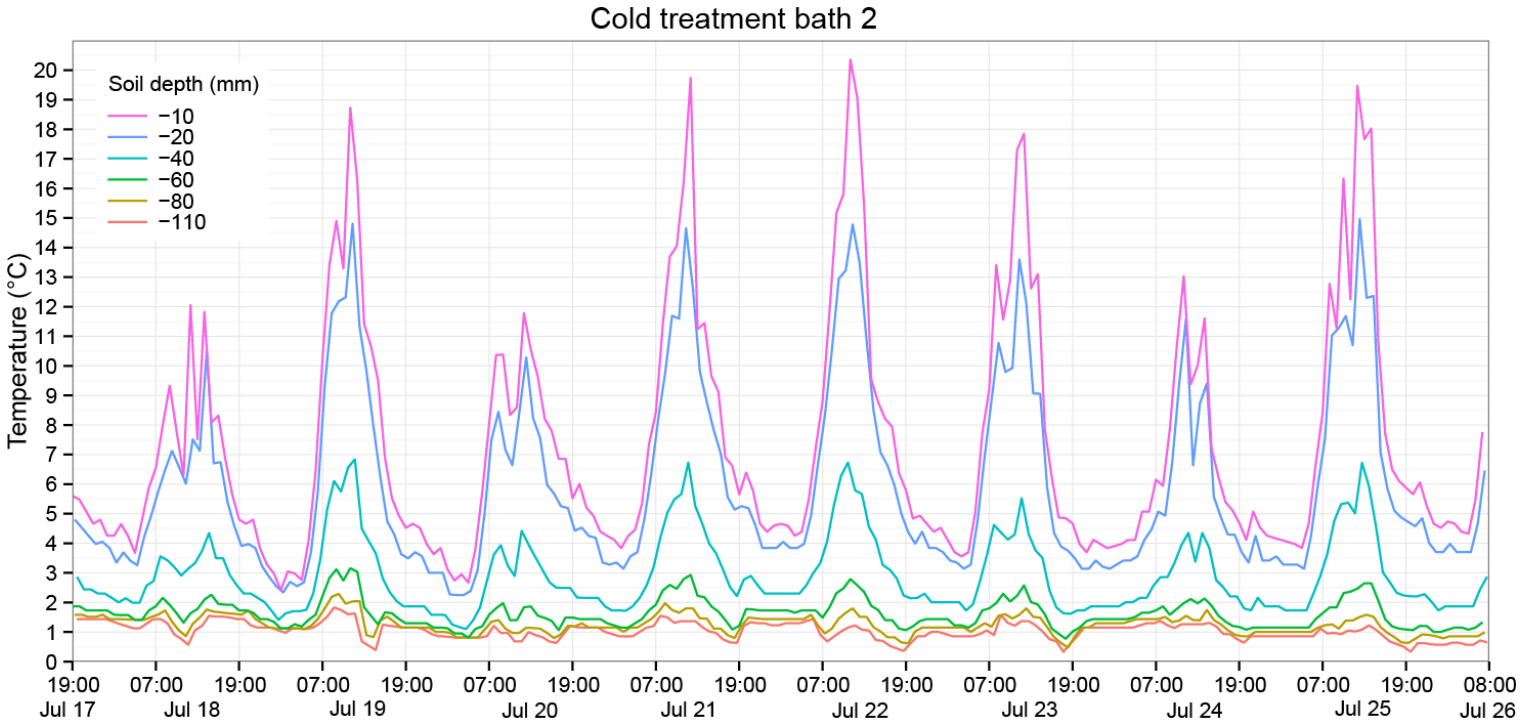
8.2 Supplementary material to Chapter 4

Figure S4.1



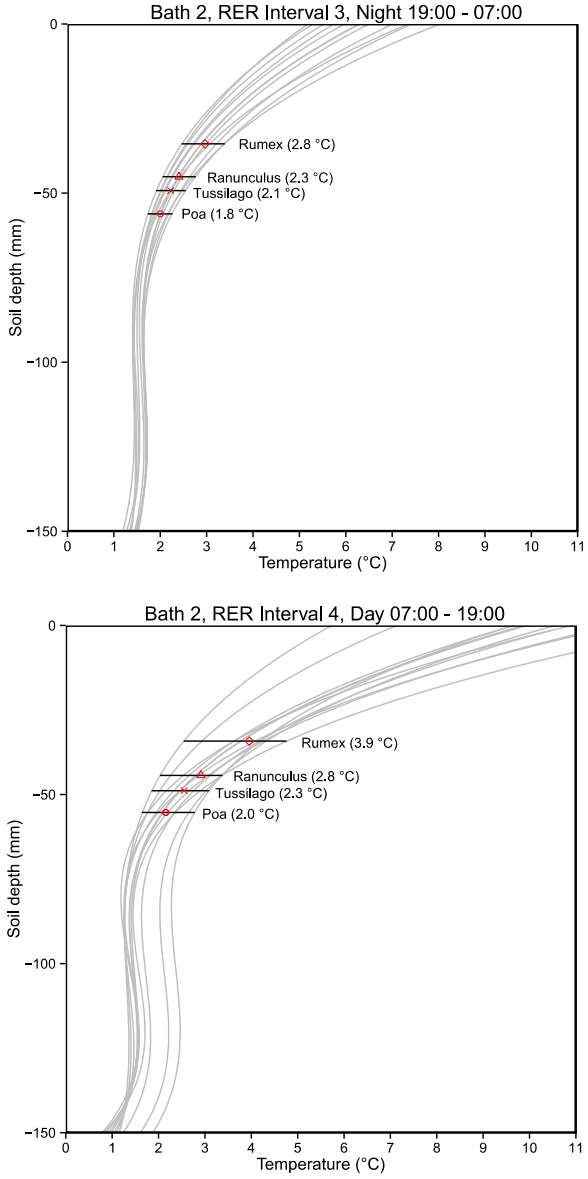
Frequency distribution of the hourly soil temperatures in the six sensor depths during the 29-treatment-days (no roots at sensor depth of -140 mm, not shown). Note, the few warmer hours in cold bath 3 (red circles) caused by cooling system failure which occurred before root elongation (RER) measurements started.

Figure S4.2



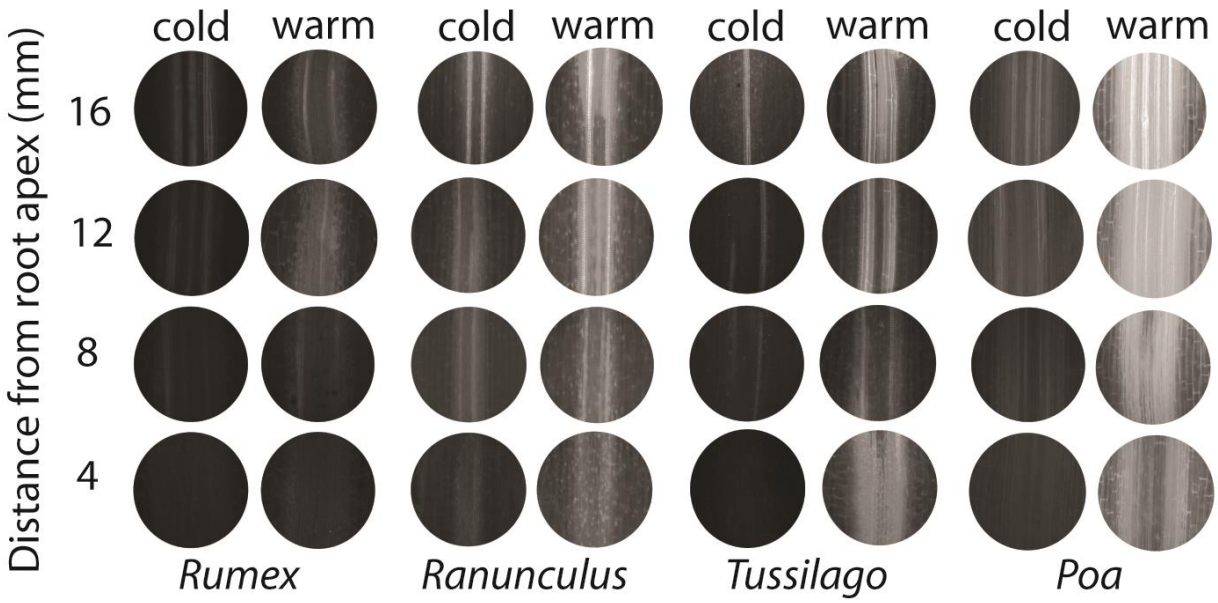
Temperature course of the seven temperature sensors in the cold treatment cylinders (bath 2) during the period of root elongation measurements (RER). Note, no roots were measured in the two uppermost (warm) soil layers of the cold bath, but these temperatures indicate that above-round plant tissues were exposed to typical alpine climate conditions (high radiation, cool nights).

Figure S4.3

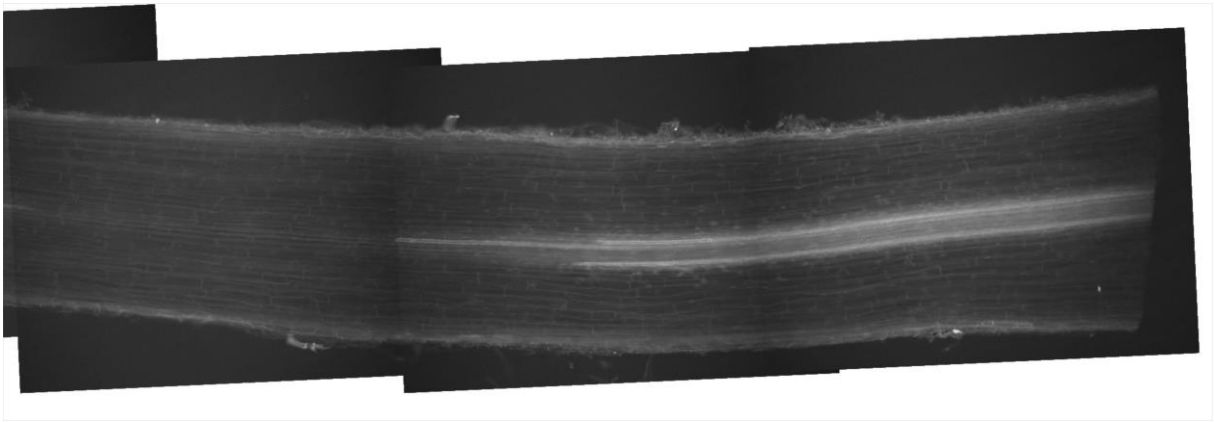


Estimation of mean root tip temperature for a RER $12h^{-1}$ from soil depth and soil temperatures. Grey functions show the 12 fits for a RER $12h^{-1}$ based on polynomial regressions of soil temperatures (measured hourly by T resistors at seven soil depths) and soil depths. Upper graph shows an example of RER $12h^{-1}$ with night hours (19:00-7:00), lower graph with day hours (7:00-19:00). Red symbols show the resulting mean root tip temperature (value in parenthesis) and black bars indicate the coldest and warmest hourly temperatures for the selected RER $12h^{-1}$.

Figure S4.4



Snap shots of 0.6 mm diameter of longitudinal cuts of the central cylinder of warm and cold treated root tips. Xylem elements were stained by berberine hemi-sulfate and aniline blue. The lower intensity of the fluorescent signal in the xylem of cold treated roots indicates a reduced lignification. HSL colour space images with the lightness values from the L channel were converted here to black-white images.



Longitudinal thin section with florescent xylem (Berberin-Hemisulfat staining) in the central cylinder. Snapshots above were taken from such images of the thin sections.

9 Acknowledgements

I would like to express my sincere gratitude to all people that I had the pleasure to work with during my doctoral thesis. First, I would like to say a big thank you to Christian Körner and Achim Walter for initiating this project. I am deeply thankful for the trust and freedom you gave me, the inspiring and motivating working environment you created, for your fruitful ideas and for financing of my work without the support of external research funds. It was a great pleasure for me to work in both working groups, the former plant ecology group in Basel and especially the crop science group in Zurich. I am thankful for the great teamwork and the inspiring discussions I was part of during the last years.

My PhD would have not been possible without the enormous commitment and help of some very important people. I would like to express my gratitude to Erika Hitbrunner for the critical and very helpful contributions to my experiments, my data analysis and most important, I appreciate the valuable inputs to the publications. Moreover, I would like to thank I Nobert Kirchgessner for the great support and the elegant solution for any problems at any time I had with image analysis. Other important people who deserve many thanks are Steven Yates and Christoph Grieder for their great support in statistics and R programming and Hansueli Zellweger and Brigitta Herzog for the field and climate chamber management. Finally, I would also like to acknowledge the help of my bachelor and master students, which I was allowed to supervise during my time at the ETH as well as the support of the civil servants at the alpine research station ALPFOR. Thank you, Maya Hildpold, Helena Rupp, Anderas Büchi, Flavian Tschur, David Muff und Lukas Müller-Eggimann.

It was a great pleasure for me to being surrounded by great colleagues. Thank you Johannes Pfeifer, Tino Colombi, Chantal Le Marie, Frank Liebisch, Kang Yu, Cordula and Michale Friedli, Lukas Kronenberg, Jonas Anderegg, Luisa Last and Marianne Wettstein for the productive collaborations and the nice off working time.

Last but not least I would like to thank my family and close friends, Elisabeth, Werner and Daniel Nagelmüller and Johanna Wegener for the support in any situation during this PhD.

10 Curriculum Vitae

Personal data

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Email	sebastian.nagelmueller@usys.ethz.ch
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Birthday	04. April 1987, Munich
Nationality	German

School education

1993–2006	Rudolf Steiner School Gröbenzell
2006	Abitur Gymnasium Olching (Final grade: 3,3)

Civil service

2006 – 2007	At Sanatorium Tannererhof, Bayrischzell, Germany

University

2007 – 2010	Bachelor of Science Biology Ludwig-Maximilians University Munich
2010	Bachelor of Science (Final grade 2,56)
2010	Bachelor Thesis: „DNA-sequence polymorphism in African and European *Dorsophila melanogaster*“
2012	Master of Science Biology: Munich Graduate Program for Evolution, Ecology and Systematics Ludwig-Maximilians University Munich (Final grade 1,75)
2012	Master thesis project at University of Basel: „The effect of low soil temperatures on root growth and morphology of glacier forefield plants in the Swiss central Alps“
2013 – 2017	PhD-Student in a cooperative project between the University of Basel, Institute of Botany, Group of Plant Ecology with Prof. Christian Körner and the ETH Zurich, Institute for Agricultural Science, Group of Crop Science with Prof. Achim Walter Title: „Low temperature limitation of plant growth, a comparative experimental approach using cold adapted alpine plants and winter crops“

Extracurricular Courses

2009	Geobotany- and forestry excursion to northern Finland with the Technical University of Munich
2010	Alpine ecology excursion to Swiss national park with LMU
2011	Alpine Botany excursion to research station Obergurgl, University of Innsbruck, Austria with LMU
2010 – 2012	Teaching at LMU: “Systematik und Pflanzenbestimmung”
2012	Zurich-Basel Plant Science Center, PhD-Summer school: „Dealing with Global Change in the Swiss Alpes“

Research experience

2009	Research internship in aquatic biology at the limnological research station, LMU, Seon, Germany.
2010	3-month molecular lab experience, bachelor thesis.
2011	2-month research internship in aquatic ecology studying the effect of micro plastic in aquatic freshwater ecosystems with Prof. Christan Laforsch, LMU, Munich.
2012	3-month research project master thesis, at the Alpine Research Station ALPFOR, Furkapass, Switzerland.
2012	Assistance at: „Hotspot Biodiversität Furka“, Furkapass, Switzerland.
2013	4 month PhD research project at ALPFOR
2014 – 2016	Development of high resolution image based leaf growth measurments in the filed at the ETH Research Station Lindau-Eschikon, Switzerland.

Presentations

2011	Nagelmüller, S., Gottschling, M. Floral Ontogeny in *Tiquilia* 2011. Poster at EES Conference, Munich, Germany
2011	Nagelmüller S, Imhof H, Laforsch C, Mirco plastic in freshwater environments 2011. Presentation at EES Conference, Munich, Germany
2016	Nagelmüller S, Walter A, Körner C. Low temperature limitation of plant growth, 2016. Oral presentation at Biology 16 Conference, Lausanne, Switzerland
2016	Nagelmüller S, Walter A, Körner C. Genotypic variations of leaf growth response to low temperature in winter cereals grown under field conditions, 2016. Poster presentation at Plant Biology Europe Congress, Prague, Czech Republic

Publications

2014 Gottschling, M., Nagelmüller, S., & Hilger, H. H. (2014). Generative ontogeny in Tiquilia (Ehretiaceae: Boraginales) and phylogenetic implications. Biological Journal of the Linnean Society, 112(3), 520-534.

2016 Nagelmüller S, Hiltbrunner E, Körner C. (2016) Critically low soil temperatures for root growth and root morphology in three alpine plant species. Alpine Botany, 126(1), 11-21

2016 Nagelmüller, S., Kirchgessner, N., Yates, S., Hiltpold, M., & Walter, A. (2016). Leaf Length Tracker: a novel approach to analyze leaf elongation close to the thermal limit of growth in the field. Journal of Experimental Botany, 67(6), 1897-1906

2017 Nagelmüller S, Hiltbrunner E, Körner C. (2017) Low temperature limits for root growth in alpine species are set by cell differentiation. *AoB PLANTS* 9:plx054. doi:10.1093/aobpla/plx054

2017 Submitted manuscript: Nagelmüller, S., Yates, S. & Walter, A. (2017) Diel leaf growth of rapeseed at critically low temperature under winter field conditions.