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# Non-destructive measurement of soybean leaf thickness via X-ray computed tomography allows the study of diel leaf growth rhythms in the third dimension

Johannes Pfeifer[1] · Michael Mielewczik[2] · Michael Friedli[3] · Norbert Kirchgessner[1] · Achim Walter[1]

**Abstract**   Present-day high-resolution leaf growth measurements provide exciting insights into diel (24-h) leaf growth rhythms and their control by the circadian clock, which match photosynthesis with oscillating environmental conditions. However, these methods are based on measurements of leaf area or elongation and neglect diel changes of leaf thickness. In contrast, the influence of various environmental stress factors to which leaves are exposed to during growth on the final leaf thickness has been studied extensively. Yet, these studies cannot elucidate how variation in leaf area and thickness are simultaneously regulated and influenced on smaller time scales. Only few methods are available to measure the thickness of young, growing leaves non-destructively. Therefore, we evaluated X-ray computed tomography to simultaneously and non-invasively record diel changes and growth of leaf thickness and area. Using conventional imaging and X-ray computed tomography leaf area, thickness and volume growth of young soybean leaves were simultaneously and non-destructively monitored at three cardinal time points during night and day for a period of 80 h under non-stressful growth conditions. Reference thickness measurements on paperboards were in good agreement to CT measurements. Comparison of CT with leaf mass data further proved the consistency of our method. Exploratory analysis showed that measurements were accurate enough for recording and analyzing relative diel changes of leaf thickness, which were considerably different to those of leaf area. Relative growth rates of leaf area were consistently positive and highest during 'nights', while diel changes in thickness fluctuated more and were temporarily negative, particularly during 'evenings'. The method is suitable for non-invasive, accurate monitoring of diel variation in leaf volume. Moreover, our results indicate that diel rhythms of leaf area and thickness show some similarity but are not tightly coupled. These differences could be due to both intrinsic control mechanisms and different sensitivities to environmental factors.

**Keywords**   Circadian rhythm · Diel leaf growth pattern · Growth monitoring · Imaging · Leaf volume · Plant phenotyping

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## Introduction

In annual plants, leaves typically constitute the major fraction of a plant's biomass. The accumulation of this biomass depends on the development of leaf structure and function, which in turn are subject to the environmental conditions, to which leaves are exposed during growth (Ainsworth et al. 2005; Chabot and Chabot 1977; Friend et al. 1965; Poorter et al. 2009; Sims et al. 1998; Tardieu et al. 1999). Intrinsic metabolic control mechanisms connected to the circadian clock help the plant to align photosynthesis (Dodd et al. 2005), respiration (Matsushika et al. 2000) and

photorespiration (McClung et al. 2000) with growth and oscillating environmental conditions, such as light–dark cycles or temperature. Good matchings result in significantly faster growth, increased carbon fixation, higher chlorophyll content, biomass production and survival (Dodd et al. 2005). The circadian clock governs diel leaf growth rhythms—both in dicotyledonous plants (Dornbusch et al. 2014; Poiré et al. 2010; Ruts et al. 2012a, b) as well as to a somewhat lesser extent in monocotyledonous plants (Caldeira et al. 2014; Poiré et al. 2010).

Any analysis of the metabolism or regulation of the growth processes and of the interplay of these processes with environmental conditions and fluctuations requires a precise, high-resolution determination of the magnitude and temporal dynamics of these processes. Such analyses have been in the focus of research efforts for a long time. However, only recently, several robust, automated and non-destructive phenotyping methods based on optical image analysis have been developed, which allow recording and analysis of diel growth patterns in the laboratory (Caldeira et al. 2014; Friedli and Walter 2015; Schmundt et al. 1998; Walter et al. 2009; Wang et al. 2011) and in the field (Mielewczik et al. 2013; Nagelmüller et al. 2016) at a high spatial and temporal resolution. Using such methods, valuable insights into intrinsic control mechanisms underlying diel leaf growth patterns could be achieved (Caldeira et al. 2014; Dornbusch et al. 2014; Poiré et al. 2010; Timm et al. 2012; Wiese et al. 2007). Yet, those methods are either based on the measurement of leaf area or leaf elongation. Unfortunately, it is not possible to record the diel rhythms of leaf biomass growth directly, because non-destructive methods for the measurement of leaf biomass are not available at the required precision.

A promising approach in this context is to consider leaf volume (Muller et al. 2007). In previous studies, authors have assumed that leaf thickness can be neglected in the calculation of the leaf volume for monocots, because they found that the thickness of the leaf blade is rather constant along age and the distance from the leaf base (Muller et al. 2007). However, even though this is a reasonable general simplification considering monocot leaf volume for several kinds of investigations, this assumption cannot be generalized for monitoring of leaf thickness in dicot plants on a small temporal scale. First, it is well known from studies on mature dicot leaves, that several environmental factors can directly and significantly influence leaf thickness on a short temporal scale (Meidner 1952). Second, there are several indicators and indices such as leaf mass area index (LMA) that seem to highlight that leaf thickness of dicot leaves might show fluctuating differences on a diurnal scale (Sims et al. 1998; Tardieu et al. 1999). Third, while monocot leaf thickness appears to be mostly constant over leaf age (Muller et al. 2007), most likely because leaf thickness

is defined at a very early stage of development (Narawatthana 2013), leaf thickness of dicot plants is very slowly but steadily increasing during the development and might show diel growth patterns. Finally, even though monitoring of leaf thickness in dicot leaves seems to be more relevant than in monocots, even in monocots, in which diurnal variation of leaf thickness remains practically unstudied, there are a few indirect indications in the literature that small fluctuations over short time-scales can occur (Fensom and Donald 1982). Furthermore, it is also known, that in monocots, thickness of leaves from different positions differs (Sant 1969). From this perspective, a non-invasive method that would allow monitoring of diurnal changes in leaf thickness during growth would be desirable.

Leaf thickness, in general, is known to be very closely correlated to leaf fresh mass per unit leaf area, leaf dry mass per unit leaf area (Friend et al. 1965; Witkoswki and; Lamont 1991), leaf water content per unit leaf area (Sims et al. 1998) and thus also to specific leaf area (SLA, projected leaf area per unit leaf dry mass, Poorter et al. 2010; Vile et al. 2005; Wilson et al. 1999). Consequently, leaf volume is more closely related to leaf biomass, at least to leaf fresh mass, than leaf area. For this reason, the measurement of leaf volume can help to investigate under which conditions it is possible to derive an estimate of leaf biomass growth from leaf area.

However, it remains to be elucidated in detail how accumulation of fresh weight, dry weight and leaf volume are coupled and regulated on a shorter time scale.

Dicotyledonous (Dale 1964; Maksymowych 1959, 1973; Sant 1969; Tichá 1985; Verbelen and De Greef 1979) plants increase leaf thickness during growth. In dicot leaves, relative expansion of leaf thickness is most pronounced at very early phases after emergence. This phase of rapid expansion corresponds to a rapid expansion of palisade cells, development of intercellular spaces and an increase in the number of cell layers (Maksymochwych 1973; Tichá 1985). After this initial increase the further expansion in leaf thickness is very slow but still present (Tichá 1985) and overall much slower than the relative increase in area, thereby enforcing the flattened leaf morphology (Kalve et al. 2014).

Moreover, final leaf thickness of fully expanded leaves can be affected by the environmental conditions during growth, such as light intensity (Poorter et al. 2009), salt stress (Robinson et al. 1983; Rozema et al. 1987), potassium availability (Battie-Laclau et al. 2014), high temperature amplitudes between night and day (Chabot and Chabot 1977) or $CO_2$ concentration (Sims et al. 1998). SLA can change during growth in both, dicotyledonous and monocotyledonous leaves and varies even among different zones of the same leaf (Tardieu et al. 1999).

On a short timescale, though, it is not known whether the increase in leaf thickness is affecting or even

counterbalancing the increase in leaf area, for which the rhythmicity of expansion processes has been reported. Tardieu et al. (1999) emphasized that SLA can be significantly increased if the relative leaf area growth is stronger limited by environmental influences than photosynthesis. Moreover, SLA can even vary throughout a diel cycle (Tardieu et al. 1999). To the best of our knowledge, detailed analyses on the temporal dynamics of those effects on a diel basis are lacking, but would be required to increase our understanding of these interactions. Yet, it is difficult to analyze the thickness or the volume of the leaf by classical approaches (Sims et al. 1998; Vile et al. 2005).

One classical approach to monitor the thickness of leaves is the use of calipers or leaf clips, which are clamped (often by strong magnets) to a point of the leaf (Bramley et al. 2013; Gebbers 2014; Meidner 1952; Sharon and Bravdo 2001). However, these clips are constructed for the measurement of leaf turgor changes of fully expanded leaves (e.g. of trees) rather than for the monitoring of leaf thickness development within delicate, growing leaves. If clamped on young, growing leaves, it is likely that the applied pressure and considerable shadowing of the studied leaves may adversely affect the thickness development during organogenesis. Moreover, point measurements do not reflect the average thickness of the whole leaf. Alternatively, destructive measurements, for example by microscopic study of cut leaf-segments, do not allow continuous measurement of the same leaf at successive points in time.

Due to the technical difficulties associated with measurements, only very few studies so far have investigated changes in leaf thickness on a diurnal scale, all requiring physical contact to the leaf. Syvertsen and Levy (1982) and Kadoya et al. (1975), for example, used linear variable displacement transducers (LVDTs), while Bachmann (1922), Chaney and Kozlowski (1969), Tyree and Cameron (1977) and Burquez (1987) applied mechanical leaf thickness change meters, and Rozema et al. (1987) utilized rotation potentiometers. However, most of these methods were only used to determine diurnal thickness changes in mature leaves, but not of young leaves during growth.

It was the aim of this study to evaluate and validate the possibility of applying X-ray computed tomography to measure leaf area, volume and thickness of young, growing leaves simultaneously, non-invasively and with sufficient precision, in a manner that takes the whole leaf into account and does not require any contact with the leaf. We also explored, if the new method can be used to acquire diel growth patterns and to investigate, to what extent diel changes and growth of leaf thickness corresponds to the relative increases in leaf area.

## Materials and methods

Soybean was used, because the authors have considerable experience with respect to areal leaf growth dynamics of this species (Ainsworth et al. 2005, 2006; Christ et al. 2006; Friedli and Walter 2015; Mielewczik et al. 2013). Under ordinary, favorable climate chamber conditions, as applied in the present work, soybean grows with a pronounced peak of growth activity in the early morning or towards the end of the night (e.g. Friedli and Walter 2015). Lowest growth rates are registered at the end of the day, which may be counterintuitive at first glance, since at this time point carbohydrates necessary for biomass accumulation are being produced. It is crucial in these investigations to ensure that the plant is not disturbed by the measurement process. Therefore, it was necessary to restrict image acquisitions to a few measurement time points to record the rhythms: Measurements were therefore performed every 8 h throughout a period of 4 days. The time points were chosen in a way that the measurements were scheduled at 4 h prior and after the expected 'evening' trough in leaf expansion as well as during the expected maximal expansion activity at the end of the night. Hereafter, the three 8-h-periods dividing the 24-h diel cycle will be abbreviated: 'night' (period between 11 p.m. and 7 a.m.), 'morning' (period between 7 a.m. and 3 p.m.) and 'evening' (period between 3 p.m. and 11 p.m.). Measurements were performed at the following times: 7 a.m., 3 p.m. and 11 p.m. In the climate chamber, plants were illuminated from 8 a.m. to 9 p.m.

### Plant material and cultivation conditions

A prerequisite of this study consisted in the reproduction of the diel leaf growth patterns observed by Friedli and Walter (2015) in order to guarantee a well-demonstrated and typical growth pattern of soybean leaves. For this reason, the same soybean cultivar [*Glycine max* (L.) Merrill, variety 'Gallec'] was investigated, the plants had the same age at the beginning of this experiment (21 days after sowing) and the plants were cultivated under growth conditions similar to the greatest possible extent to the conditions applied in Friedli and Walter (2015). Corresponding to Friedli and Walter (2015) water retaining ceramic pots were used and plants were watered each morning at 6.45 a.m. to 95% field capacity. The light/dark photoperiod was 13:11 h (corresponding to April in Switzerland, when soybean is commonly sown), light intensity was $580 \pm 75$ µmol m$^{-2}$ s$^{-1}$ PAR, average temperature during the light period was 24 °C and during the dark period 20 °C. Relative humidity was kept constant at 60%. Although the light intensity was, compared to many other experiments, relatively high, leaves were not exposed to heat stress as the distance of the lamps to the canopy was about 80 cm. Further details,

for example about the soil used, are reported in Friedli and Walter (2015). For the experiment, 40 plants were cultivated and 10 healthy plants of similar size were selected from those for the measurements. Five replicate plants (n = 5) were used for repeated CT measurements (hereafter abbreviated: CT plants) and five replicate plants (n = 5) were used as controls (hereafter: control plants). For each plant, the middle leaflet of the second trifoliate leaf was marked with a little piece of twine to simplify repeated measurements. Exceptions, in which the growth conditions slightly differed from the conditions in Friedli and Walter (2015), were the time points in which the X-ray computed tomography scans were performed, as the plants needed to be brought from the climate chamber to the tomograph for scanning and afterwards had to be returned to the climate chamber. Although those chambers were situated in the same building on a lower floor as the CT scanner, this fact resulted in a transportation time of about 2 min. To keep control plants and CT plants under similar growth conditions, the control plants were also brought out of the climate chamber and placed next to the tomograph while the CT plants were scanned. Moreover, the air temperature near the plants was kept as constant as possible during the time of plant transport to the tomograph and their return to the climate chamber, as all plants (controls and CT plants) were placed in an insulating Styrofoam box containing some thermal packs at the bottom of the box on which the plants were placed. Box and thermal packs were placed in the climate chamber beforehand, for several hours, in order to let the thermal packs reach the ambient temperature of the climate chamber. Furthermore, the light conditions were also mimicked as the plants were illuminated with additional light at day and were kept in the dark as well as possible during transport and measurement during the night.

## X-ray computed tomography (CT)

X-ray CT scans were performed at the Swiss Federal Institute of Technology Zurich (ETH Zürich, Switzerland) using a phoenix v|tome|x s 240 X-ray scanner equipped with a GE DXR250 HCD (high contrast detector, GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) at a voxel size (with binning 2×2) of 0.05 mm voxel edge length. To prevent movement of scanned leaves during X-ray image acquisition leaves were gently fixed in a custom-made mount assembled of light plastic foam (Supplemental Fig. S1). For this purpose, the leaves were slightly pressed together for just some millimeters without exerting a force to the midvein and they were inserted carefully into the mount. During the scan, the mount and sample rotate slowly for 360° while they are penetrated by the X-ray beam. After the beam passes the sample, it hits

**Table 1** Acquisition parameters for X-ray computed tomography

| | |
|---|---|
| Scanning parameters | |
| Voxel size with binning (mm) | 0.05 mm voxel edge length |
| Binning | 2×2 |
| Current (µA) | 100 |
| Voltage (kV) | 20 |
| Number of images per scan | 1600 |
| Averaged images | 1 |
| Skipped images | 0 |
| Filtering | 0.1 mm aluminum |
| Observation ROI option | No |
| Exposure time per image (ms) | 200 |
| Scan duration (s) | 320 |
| Multiscan | No |
| Fastscan | Yes |
| Reconstruction parameters | |
| Downscaling to unsigned 16 bit | Yes |
| Auto scan optimizer | Automatic |
| Beam hardening correction | Correction value of 4, assuming different materials |
| Ring artefact correction | No |

the detector unit with which a large number of images (here 1600) are acquired for subsequent 3D reconstruction.

It is well known that strong X-ray doses above 15 Gy can lead to oxidative stress due to formation of free radicals in plants (Riley 1994; Peréz-Torres et al. 2015). For this reason, acquisition parameters for tomography (Table 1, for further explanations to CT image analysis see Pfeifer et al. 2015) were optimized in an additional, preliminary experiment with the aim to minimize the radiation dose leaves had to be exposed to. The calculated maximum absorbed dose was 0.0027 Gy per scan of 5 min using the following formula: Absorbed dose (Gy) = dose rate (Gy s$^{-1}$ µA$^{-1}$) × time of exposition (s) × current (µA), where the dose rate is calculated at 20 kV and a distance of 20 cm between the X-ray source and the center of the leaf (further information on calculations by GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany). Consequently, plants were exposed to a maximum of 0.03 Gy in this experiment (eleven scans within 80 h).

Volumes were reconstructed using the software datos|x (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany). For reconstruction (in 32-bit float format) no ring artefact correction, but an autoscan optimization and a beam hardening correction were performed (Table 1).

## Volume data analysis

CT volumes were analyzed by Visual Studio Max 2.2 software (Volume Graphics GmbH, Heidelberg, Germany) with

the add-on modules "Coordinate measurement" (advanced surface determination) and "Wall thickness analysis" (for further explanation see Pfeifer et al. 2015). Original images (32-bit float) were downscaled to unsigned 16-bit format. Data was filtered by a median filter of $5\times5\times5$ voxels (voxel gray values replaced by median gray value of neighboring voxels). This procedure preserves edges while artefacts due to scattering can be diminished. In the first step, all plant structures were segmented using the common surface determination tool (manual selection of air as background and leaf as material in "define material by example area"-function). A new region of interest (ROI) was generated. Next, the petiole was manually erased in this ROI. Next, the ROI was split into separate sub-ROIs. The sub-ROI containing the leaf blade was dilated for five voxels and extracted into a new volume. In this volume containing the leaf voxels and the layer of five neighboring voxels around the leaf voxels (Fig. 1), the leaf could be segmented very precisely (with sub-voxel-accuracy) by applying the advanced surface determination tool. The advanced surface determination refines the surface locally at several thousand locations along the leaf surface by a local adjustment according to the gradient of the gray values. The same gray value is reinterpreted according to the gray value of the neighboring voxels. Frequency distributions of leaf thickness were determined using the add-on "Wall thickness analysis". Similar to surface calculation performed with the advanced surface determination algorithm, the thickness of the leaf was calculated at several thousand locations across the leaf surface (Fig. 2). The surface determined by the advanced surface determination tool serves as the starting contour. All image processing steps took about 6 min per leaf including data loading and filtering. Leaf thickness distributions were collected (example shown in Supplemental Fig. S2) but were not further analyzed in the context of this feasibility study. The obtained thickness distributions are nearly normally distributed (Supplemental Fig. S2), which is an indicator for the accuracy of the method. Moreover, the plausible spatial distribution of the thickness along the leaf dimensions (including thicker veins and thinner intercostal tissue) in Fig. 2 is an additional indicator of the measurement accuracy and spatial plausibility.

## Leaf area measurement by digital imaging

Prior to the CT scans, leaves from CT plants and control plants were imaged by a digital single-lens reflex (DSLR) camera (18 Mpx, with a 28 mm electro focus (EFS) fixed lens, EOS digital 550D; Canon U.S.A., Inc., Melville, NY, USA) mounted on a tripod. The leaf position was adjusted using a laboratory lifting platform and the leaves were gently flattened by placing an overhead transparency foil on top of the leaves. A ruler, which was put in the same picture, served as calibration reference. The background was equipped with blue cardboard that allowed for image segmentation with the software ImageJ (version 1.49 g, ImageJ, National Institutes of Health, Bethesda, MD, USA) using color thresholds.

## Reference thickness measurements on customary papers

Accurate thickness measurements on real leaves using microscopic approaches or calipers are very challenging, as leaves are soft and can be compressed by cutting or clamping. Therefore, the thickness of different customary papers and paperboards were measured in order to validate and demonstrate the precision of the CT-based method presented here, using the above described protocol (for CT parameters see Table 1; the same voxel size of 0.05 mm was used) and a simple digital micrometer (resolution 0.001 mm, http://www.aliexpress.com, China; hereafter abbreviated with caliper) as a reference. If small leaves would be measured (e.g. leaves smaller than 1 cm$^2$), the voxel size could be decreased to a value of 0.01 mm with our used system by decreasing the distance to the X-ray source (tube), while still the entire leaf would be within the field of view. Supplemental Fig. S3 shows thickness measurement data from seven different customary papers, measured with CT scans performed at different resolutions (=voxel edge lengths) down to 0.01 mm.

## Statistical analysis

Relative growth rates (RGR) from two consecutive images (in case of photos) or CT-volumes, respectively, were computed using the equation used by Friedli and Walter (2015): RGR (in % per hour) $= ((\ln A_2 - \ln A_1)/(t_2 - t_1))\times100$, where $A_1$ is the leaf area (or volume) at time $t_1$ and $A_2$ is the area (or volume) at time $t_2$. The term $t_2 - t_1$ always equaled 8 h in this analysis.

Unpaired $t$ tests (two tailed) were performed with the SPSS statistical software package (version 17.0, SPSS Inc., USA). Plotting of figures was performed using the statistical software package SigmaPlot 12.5 (SigmaPlot, Systat Software Inc., Richmond, CA, USA) and R (R ver. 3.1.3, R Development Core Team, Vienna, Austria) and the R package "BlandAltmanLeh" to create Bland–Altman plots (Bland and Altman 1986; Lehnert 2015). Median leaf thickness was calculated from leaf thickness distributions using MatLab 8.0 (The Mathworks, Natick, Massachusetts, United States; script provided as an .exe-file in Supplemental file 1), because the median is more robust then the arithmetic mean in the presence of possible outliers. Leaf volumes were calculated in two ways: first, by multiplication of the median of thickness with CT area, and second, by

**Fig. 1** Precise determina-
tion of the leaf surface. In the
volume generated by dilation
(see "Materials and methods"
section), containing the leaf
voxels (*light gray values*) and
the layer of five neighbor-
ing voxels around the leaf
voxels (*dark gray values*) **a**, the
advanced surface determina-
tion tool **b** was applied. In **a**
a strongly magnified part of a
cross section through the leaf
is shown. As the histogram
used for the advanced surface
determination tool contains only
*gray* value data from leaf voxels
and air voxels, the automatic
surface determination could be
used. This application computes
the background *gray* value
(representing air voxels) and the
material *gray* value (represent-
ing leaf voxels) in an automated
fashion. The result of the auto-
mated surface determination
is visible as a *thin yellow line*
in **a**, while the preview of the
advanced surface determination
is represented by a *thick yellow
line*. The newly determined
surface serves—in the next
step—as a starting contour to
produce a new region of interest
(ROI) and to compute the thick-
ness values over the entire leaf
surface
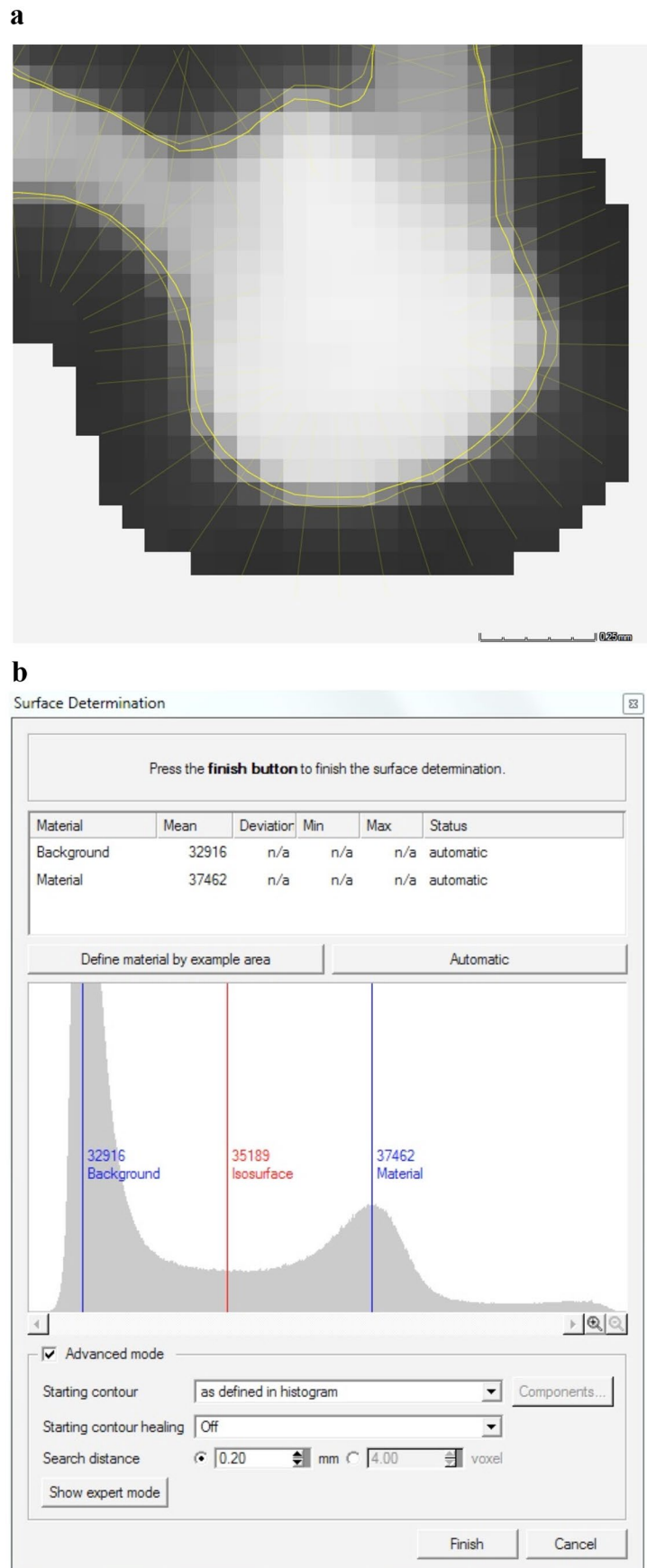
**Fig. 2** Leaf thickness was measured over the entire leaf area with the add-on module "Wall thickness analysis" of VG Studio Max 2.2. In a cross section of a leaf **a**, for which the position of the section is indicated by a *blue plane* in the 3D view **b**, representative thickness values are visible, showing for example relatively thin regions between the leaf veins (*orange/red*) and the relatively thick midvein (*pink*)
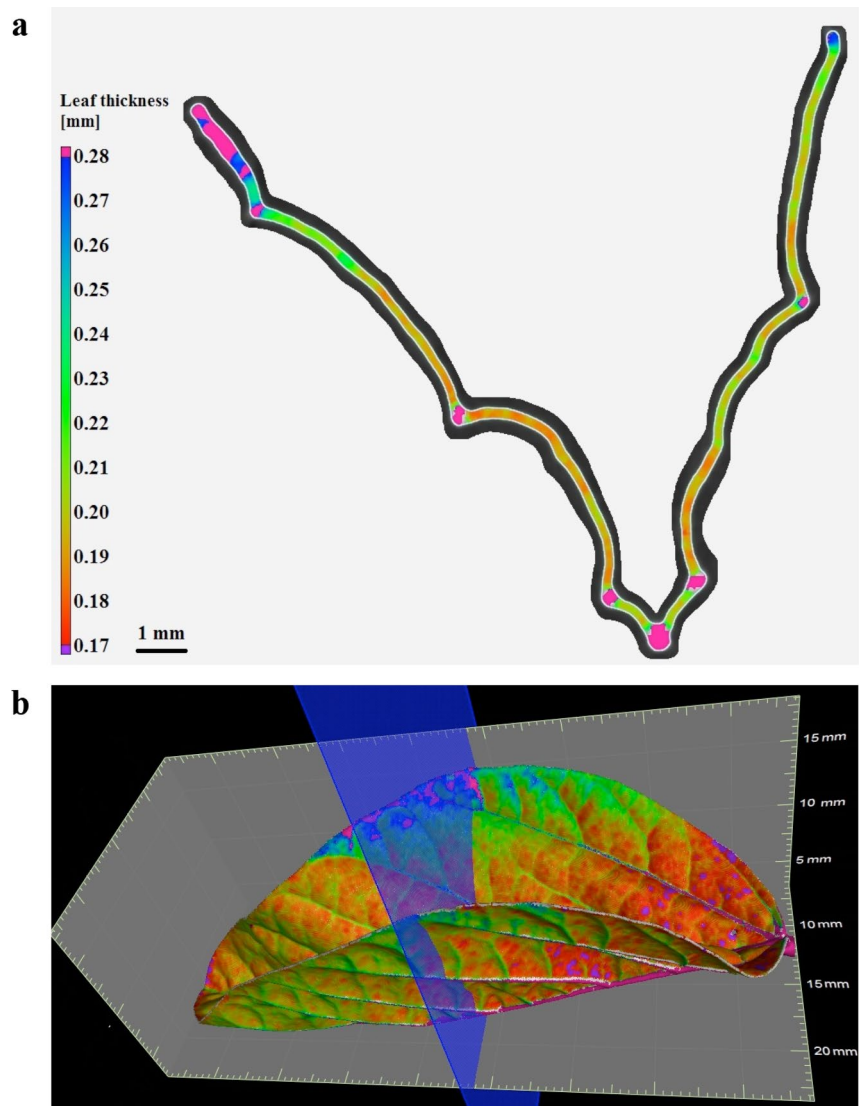


direct extraction of the volumes given by VG Studio Max 2.2.

## Results

### Validation of measurements by X-ray computed tomography (CT)

The thickness of customary papers and paperboards was strongly and significantly correlated with the thickness measured with a caliper ($R^2 = 0.9790$, $P < 0.001$; Fig. 3b). Thickness values obtained by CT were slightly reduced compared to the values measured with the caliper, showing a small bias of −0.015 mm, when thin papers were included in the modified Bland–Altman plot (Fig. 3d). If only the thickness from papers thicker than 0.15 mm were considered (the relevant range of the studied leaves, data

not shown separately), the average difference (bias) of the two methods was reduced to −0.002 mm, and the values were much more symmetrically distributed close to zero difference.

Leaf area values obtained by CT were strongly and significantly correlated with the leaf area extracted from photos ($R^2 = 0.9954$, $P < 0.001$; Fig. 3a). As visible in the modified Bland–Altman plot, the CT values were on average 0.8 cm$^2$ higher than the values from the image analysis (Fig. 3c).

### Leaf volume correlates with biomass

As an additional check of plausibility of the results, we compared fresh and dry mass of the leaves with the volumetric data measured by CT, respectively, and observed very strong correlations ($R^2 = 0.9871$, $P < 0.001$; and $R^2 = 0.9876$, $P < 0.001$; Fig. 4a, b). Leaf area from CT

**a**



**c**



**b**



**d**
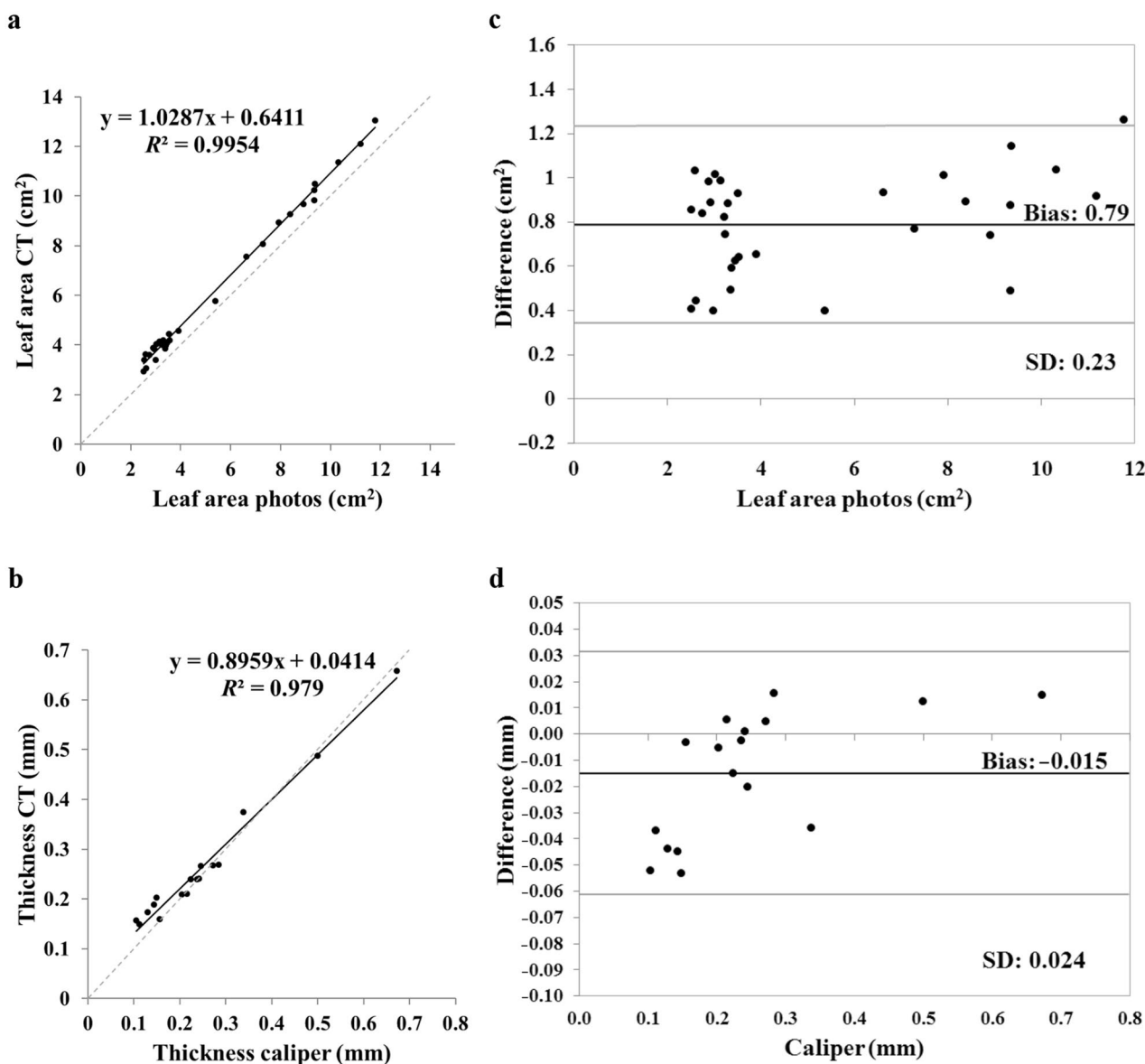


**Fig. 3** Validation of the measurement precision of the CT data. Leaf area values obtained from the CT measurements were correlated with the leaf area values obtained from photos **a** ($n=31$). For validation of thickness measurements, the CT values of different customary papers and paperboards were correlated with the thickness obtained with a caliper **b** ($n=17$). The *dashed grey lines* in **a** and **b** annotate the zero difference (perfect correlation) and visualize systematic differences. The same data from **a** and **b** were plotted in modified Bland–Altman plots **c** and **d**, where the *black horizontal lines* show the average differences (bias, indicating systematic errors) and the *dark grey lines* show 1.96 (*asterisk*) standard deviation from the mean differences for an estimation of the 95% confidence interval

measurements was also highly correlated to fresh and dry mass ($R^2=0.9822$, $P<0.001$; and $R^2=0.9828$, $P<0.001$; Fig. 4c, d). Very strong correlations were observed as well for the comparison of leaf area of the advanced surface determination from CT reconstructions (leaf area CT) and leaf volume ($R^2=0.9858$, $P<0.001$; Fig. 4e) as well as for leaf area from optical measurements (leaf area photos) and leaf volume ($R^2=0.9853$, $P<0.001$; Fig. 4f).

## Leaf area, leaf thickness and leaf volume growth

Both plants measured by CT and control plants showed a total leaf area growth (125 and 161%, respectively) in the order of magnitude previously observed (127%) by Friedli and Walter (2015) within the 80 h period of measurements (Supplemental Fig. S4a). No differences in development and habitus could be observed between CT
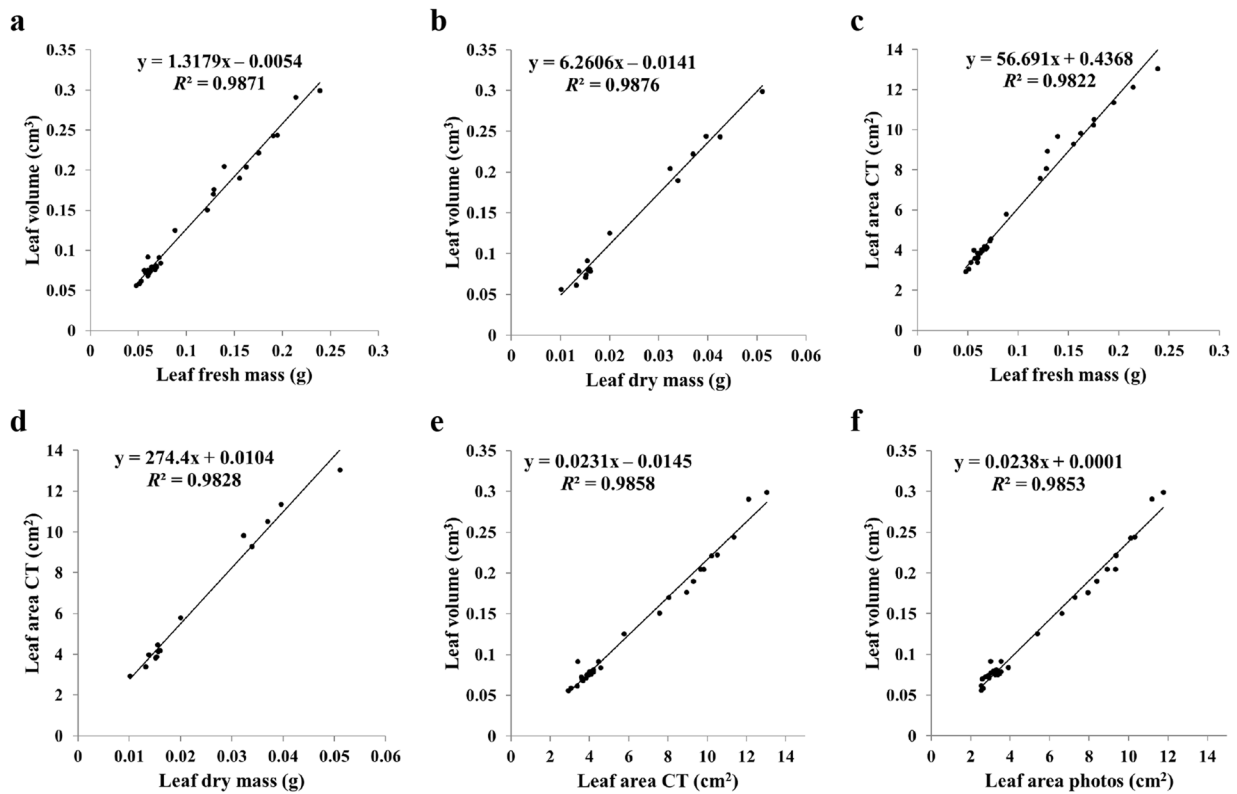
**Fig. 4** Validation of CT analysis data. Correlation between leaf fresh mass (**a**) ($n=33$) and leaf dry mass (**b**) ($n=17$) respectively, with the volume data measured by CT (leaf volume). Correlation between leaf area CT and leaf fresh mass ($n=32$) (**c**) and leaf dry mass (**d**) ($n=17$), respectively. Correlation between leaf area CT (**e**) ($n=32$) and leaf area photos (**f**) ($n=33$) respectively, and leaf volume. All $P$ values were smaller than 0.001

plants and controls at the end of the experiment (Supplemental Fig. S6), and leaf fresh mass was not significantly different (Supplemental Fig. S4b; Unpaired $t$ test, two-tailed $P$ value $=0.25$).

The average absolute leaf thickness growth within the duration of the experiment (12%) was about ten times smaller compared to the average absolute leaf area increase (125%) within the same period (Fig. 5a). Already the absolute measures of thickness showed clear fluctuations between day and night, whereby the ratio area and thickness were not identical over time and instead showed a slight diel uncoupling (Supplemental Fig. S5).
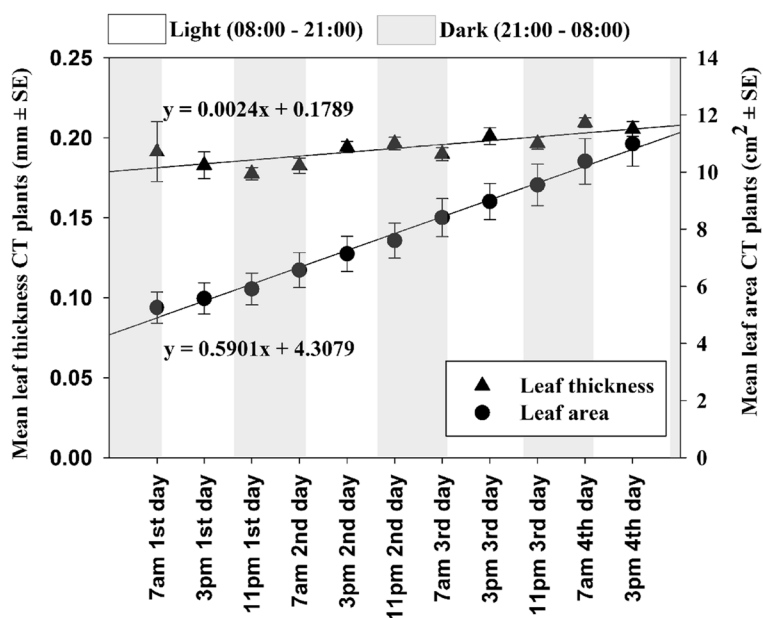
Moreover, all plants showed, with respect to leaf area (Fig. 6a), the relative diel leaf growth patterns previously observed by Friedli and Walter (2015). The rhythm observed by Friedli and Walter (2015) matched better with the rhythm of the area growth data measured by CT than the area growth data measured by digital imaging (Fig. 6a). Highest leaf area growth rates were consistently observed within the 'nights', while lowest growth rates were consistently observed during the 'evenings'. This shows that the leaves scanned several times by X-rays grew normally.

Similar to leaf area growth, leaf volume growth rates, measured by X-ray scanned plants, were smallest during the 'evenings' (Fig. 6b). However, at two of the 3 days of observation, maximum volumetric growth was observed during the 'mornings'. When growth rates of all leaves and all 3 days were averaged (Fig. 6c), it became obvious, that leaf thickness did not increase in the 'evenings' (in contrast to area) but showed a substantial increase both at 'nights' and during the 'mornings'. However, even these comparatively slight changes with respect to absolute leaf thickness growth affected the leaf volume growth rates strongly enough to shift the volume growth rhythm towards the morning on 2 of 3 days, when compared to leaf area growth rhythm.

## Discussion

Our study shows that X-ray computed tomography can be used to measure both leaf area and thickness and thus volume of dicotyledonous plants simultaneously with sufficient precision even on young, growing leaves. Validation of the data shows a good agreement between

**Fig. 5** Mean values of absolute leaf thickness (mean ± SE of median of thickness distributions) and absolute leaf area values (mean ± SE) of CT plants (CT values) measured within the 80 h duration of the experiment. *Thick lines* in the abscissa represent the times of CT measurements ($n = 5$)



Considering the diel growth patterns in more detail

In previous studies, the peak of relative leaf area growth rates of young, annual dicotyledonous plants has been observed in the night and/or early morning (Ainsworth et al. 2005; Dornbusch et al. 2014; Friedli and Walter 2015; Poiré et al. 2010; Pantin et al. 2011; Schurr et al. 2000). Our results fit well to these previous findings and we observed the same pattern in our data (Fig. 6). Moreover, our data show that we achieved an accuracy in measuring leaf thickness, which was appropriate to characterize diel changes (Figs. 3, 6). Therefore, our results allow us to investigate whether the diel relative growth rates of leaf thickness show a rhythm synchronous to the diel relative growth rates of leaf area. Furthermore, we can approach the question whether diel relative leaf area growth rates are representative for diel leaf volume changes and eventually for biomass accumulation. With respect to the first question, we observed that diel relative leaf area and leaf

classical measurements and those performed using X-ray computed tomography. Our further exploratory investigation of changes in diel (24-h) growth also shows that not only growth of the leaf area, but also that of the leaf thickness with small changes occurring throughout the diurnal cycle can be performed using X-ray computed tomography. Therefore, our method appears promising to determine leaf thickness non-invasively and contactless. X-ray computed tomography thereby has several advantages compared to other classical methods which require physical contact to the leaf and thus provide a mechanical stress to the leaf and its surface. Moreover, those methods do not allow simultaneous measurement of leaf area of the same leaf.

thickness growth rhythms were not synchronous. The relative changes in leaf thickness and observed growth patterns showed several differences, when compared to the relative leaf area growth rhythm. Most striking, leaf thickness occasionally showed negative changes, the pattern was less consistent and the ranking of the growth rates between 'morning', 'night' and 'evening' was shifted towards the 'morning' during 2 of 3 days if compared to leaf area. Therefore, on the basis of this data, it appears reasonable to assume that the diel growth rhythm of leaf thickness is maybe less or probably differently controlled by the circadian clock and external factors than the diel growth rhythm of leaf area and that the two rhythms are not tightly coupled (Supplemental Fig. S5). Moreover, our data indicate that leaf thickness most likely does not depend on the growth of structural components to the same extent as area growth (Pantin et al. 2011), but fluctuates more due to other influences, such as changes in plant water potential and leaf turgor (Ben-Haj-Salah and Tardieu 1996; Bramley et al. 2013; McBurney 1992; Pantin et al. 2012; Sharon and Bravdo 2001; Seelig et al. 2015). Leaf turgor, in turn, and its spatial distribution in leaves, does not control leaf area expansion rates, as demonstrated by Schurr et al. (2000) and Schmundt et al. (1998). They showed that area expansion rates are most likely controlled by the expansibility of the cell wall. Even if the water potential was kept constant by application of pneumatic pressure to the root system, short-term changes in the leaf area growth rate were observed during the light–dark transition (Schurr et al. 2000).

The leaf area growth peak during the 'night' can be explained by an increased relative water uptake into the leaves, as evapotranspiration is at a minimum during the 'night', and the conversion of storage carbohydrates

**Fig. 6** Relative leaf area growth rates (RGR) of control plants (*n* = 5) and CT plants (*n* = 5) measured by CT or digital photos as indicated in *brackets*, and leaf area in the experiment of Friedli and Walter measured with the digital photo based DISP-method (2015) **a** Leaf volume [from VG Studio Max 2.2 and calculated with CT area (*asterisk*) median thickness], leaf thickness and leaf area measured by CT of CT plants (*n* = 5) **b** *Thick lines* in the abscissa represent the times of CT measurements (which were performed every 8 h). Boxplots (mean: *dotted line*, median: *continuous line*) of RGR of leaf thickness (non-hatched) and area (hatched) of CT plants pooled for all times of day (**c**), (*n* = 5 plants over whole experimental duration)

(starch) into leaf structural components, like cell walls is proceeding at a high rate (Pantin et al. 2011; Schurr et al. 2000). In previous studies, it was shown that long-term leaf growth patterns can be maintained even when leaves are exposed to short-term changes in environmental conditions (Poiré et al. 2010; Walter and Schurr 2005). For example, circadian rhythms of tobacco leaf area growth remained unmodified even when light conditions were changed from day-night cycles to continuous light or when day-night temperature patterns were strongly modulated (Poiré et al. 2010). Nevertheless, it is well known that leaf growth rate may respond pronouncedly even to short-term changes of environmental conditions such as sudden increases or decreases of light intensity (Walter and Schurr 2005). If environmental changes persist, such as after the onset of any stress experiment, plants will need to acclimate, will alter growth and will also alter SLA (Poorter et al. 2010) leading to alterations also in leaf thickness. Under extreme environmental conditions final leaf thickness can be affected strongly. Then, the method presented here could be used to perform non-destructive analysis of leaf thickness and leaf volume growth patterns.

## Limitations of the study

Leaf area measured by computed tomography correlated well with other established methods of area estimation (photos), even though giving slightly higher values than the reference measurements (Fig. 4 a). As visible in the modified Bland–Altman plot (Fig. 4c), the CT determined leaf area showed an average systematic offset of 0.8 cm$^2$ compared to values from the image analysis of photographs. This systematic difference may be no "error", but can be explained by the fact that the leaves are not completely flat during photo acquisition, even if gently flattened as performed here. In contrast, in the CT surface determination, the surface is adapted to the 3D structure and fits nicely to the convexity of the leaves. We noticed an attenuation of fluctuations of leaf area expansion when measured on CT rather than on photos (Fig. 6a). This effect needs further study as might originate from minute differences in segmentation techniques as well as differences in strain and
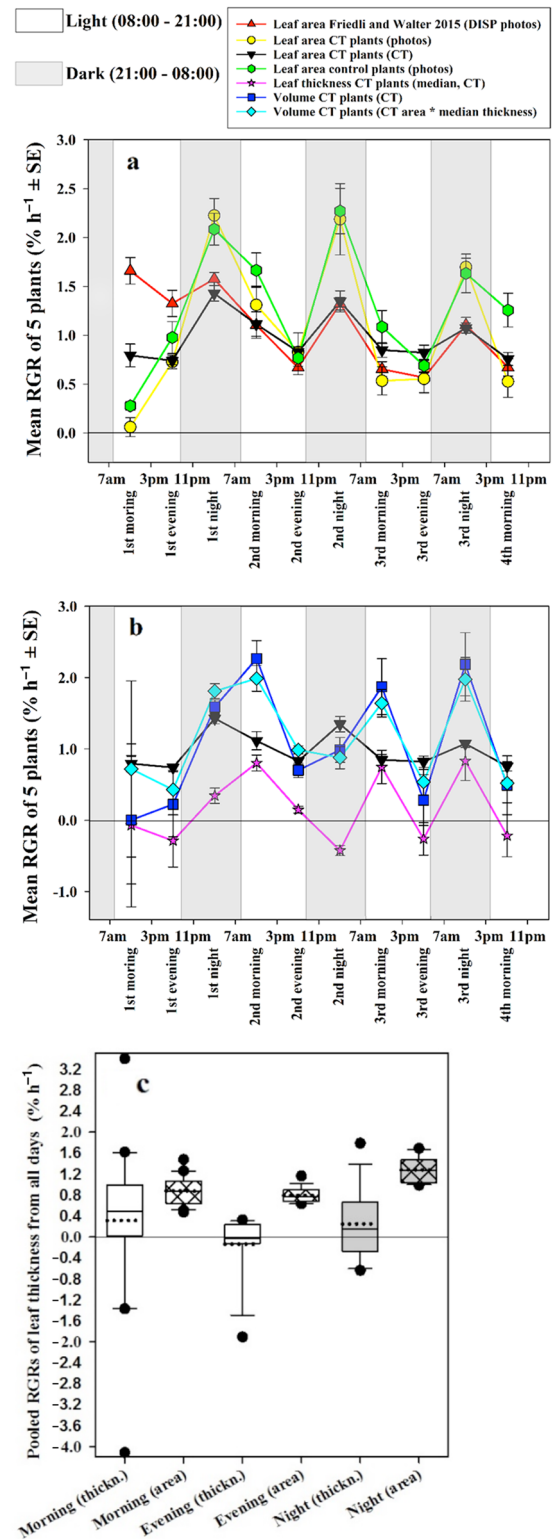
tensile forces applied to the leaves. Eventually, resolution differences between CT and photos might also cause differences in the relative fluctuation.

Thickness measurements are more demanding, though, due to the small dimension of leaf thickness. Technically,

the estimation of thickness shows good agreement and correlates well with reference measurements (Fig. 4 b, d). However, in a range below 0.15 mm thickness measurement becomes unreliable at a resolution of 0.05 mm voxel edge length. Because leaf thickness of many plant species ranges between 0.15 and 2 mm (Abrams and Kubiske 1990; Castro-Esau et al. 2006; Gausman and Allen 1973; Knapp and Carter 1998), CT seems to be well suited to estimate leaf thickness and thus also leaf volume. Nevertheless, it must be clearly stated that a leaf thickness of about 0.1 mm constitutes the absolute lower resolution limit of the CT method, using the applied device and the chosen distance between plant and X-ray beam unit (tube), due to the underlying voxel size at the chosen distance from detector and beam source. This results in some constraints in applied imaging, especially in the investigations of developing young leaves or investigations of some model plant species such as *Arabidopsis* that can have very thin leaves in the range of about 0.1 mm thickness and below (Pyke et al. 1991; Wuyts et al. 2010, 2012). Yet, those constraints are less severe than it might appear at first glance, since soybean leaves are relatively large compared to *Arabidopsis* leaves. Therefore, the plants had to be positioned relatively far away from the tube, to facilitate imaging of the entire leaf. When smaller plant leaves, such as those of *Arabidopsis* would be investigated, the plants could be positioned closer to the tube, which would decrease the voxel size (a value of down to 0.01 mm can be achieved with the here used system, see Supplemental Fig. S2). In previous studies focusing on visualization it was already shown that the leaves of *Arabidopsis* can be visualized by X-ray computed tomography rendering the leaf surface and leaf volume in excellent resolution of down to 0.0006 mm (Dhont et al. 2010).

Especially for minuscule relative changes of leaf thickness, the introduction of new CT sensor generations with slightly increased sensor resolution might greatly improve accuracy of measurements. Overall, the spatial resolution used in our experiment is essentially defined by both voxel edge length and bit rate, because gray values are considered to achieve sub-voxel-accuracy of the surface determination.

**Future investigations**

In future studies, complementary physiological measurements would be exciting, for example, to better explain the observed negative leaf thickness growth rates during the 'evening' and 'night', as shown in Figs. 5, 6). Measurements of stomatal conductance, leaf water potential, leaf turgor, transpiration rate and relative water content of the leaves could reveal underlying processes as related to diel fluctuations during growth.

With the intention to further develop methods for studying diel leaf growth patterns it would be interesting to include complementary imaging methods, such as optical coherence tomography (OCT) in future studies. Using OCT, leaf cross-sections could be analyzed non-destructively at a spatial resolution down to about 1 µm, which not only allows measurement of leaf thickness, but presumably also measurement of the leaf air content (Hettinger et al. 2000; Lee et al. 2006). The simultaneous, non-destructive measurement of leaf air content would provide the opportunity to correct the measured leaf volumes for the air content. The remaining volume would most likely even better correlate with leaf fresh mass. Indeed, measuring whole leaves by OCT will be challenging due to the restricted field of view, which is commonly in the range of a few millimeters. However, additional OCT measurements could be used to verify leaf thickness measurements and add estimations of leaf air content into a modelled leaf biomass growth monitoring.

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