

1 **Title:**

2 A new device for continuous non-invasive measurements of leaf water content using NIR-transmission  
3 allowing dynamic tracking of water budgets

4

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22 Abbreviations

23 LWC: leaf water content, NIR: near infrared, rH: relative humidity, SNR: signal to noise ratio, VPD:

24 vapour pressure deficit

## 25 **Abstract**

26 Leaf water content (LWC) permanently fluctuates under variable transpiration rate and sap flow and  
27 influences e.g. stomatal responses and osmotic adjustment of plant cells. Continuous recordings of  
28 LWC are therefore central for the investigation of the regulatory networks stabilizing leaf hydration.  
29 Available measurement methods, however, either influence local hydration, interfere with the local  
30 leaf micro-environment or cannot easily be combined with other techniques. To overcome these  
31 limitations a non-invasive sensor was developed which uses light transmission in the NIR range for  
32 precise continuous recordings of LWC. For LWC measurements the transmission ratio of two NIR  
33 wavelengths was recorded using a leaf-specific calibration. Pulsed measurement beams enabled  
34 measurements under ambient light conditions. The contact-free sensor allows miniaturization and can  
35 be integrated into many different experimental settings. Example measurements of LWC during  
36 disturbances and recoveries of leaf water balance show the high precision and temporal resolution of  
37 the LWC sensor and demonstrate possible method combinations. Simultaneous measurements of LWC  
38 and transpiration allows to calculate petiole influx informing about the dynamic leaf water balance.  
39 With simultaneous measurements of stomatal apertures the relevant stomatal and hydraulic processes  
40 are covered, allowing insights into dynamic properties of the involved positive and negative feed-back  
41 loops.

## 42 **Keywords**

43 leaf water content, stomata, transpiration, sensor, infrared

## 45 **Introduction**

46 A sufficient and stable hydration of cells is essential for aerial plant organs to survive in the often  
47 desiccating atmosphere. In the leaves of vascular plants various mechanisms have evolved to provide  
48 sufficient water uptake while restricting and regulating transpirational water loss to ensure a stable leaf  
49 water content which not only stays within safe margins for survival, but is optimal for cellular  
50 processes like photosynthesis. Maintaining a stable hydration under any combination of permanently  
51 fluctuating often adverse environmental influences requires the interaction of various sensing and  
52 regulating mechanisms tightly controlling both the uptake of water to leaves and transpirational losses  
53 through stomatal pores. Tissue water content can thus be seen as one of the most tightly controlled  
54 physical properties of vascular plants. Many of the mechanisms regulating leaf water content form  
55 negative feedback loops whereby deviations of LWC or related physicochemical properties are sensed  
56 and transformed into short, mid or long term responses aimed to recover the target state of water  
57 content. Responses range from short term adjustments (e.g. stomatal responses) over mid-term  
58 osmotic adjustments to long term growth responses leading to morphological adaptations (Osakabe et  
59 al. 2014; Bielach et al. 2017; Buckley 2019; Chérel and Gaillard 2019). Water status in these feedback

60 loops is both the controlled condition and serves as the input to the homeostatic feedback-loops.  
61 The most important and effective way to regulate leaf water content is the control of leaf transpiration  
62 by osmotically driven changes of guard cell turgor and hence pore-width. The mechanism by which  
63 guard cells are triggered to respond to changing LWC however is not yet fully understood despite of a  
64 huge amount of studies published on this topic over the last decades.  
65 Much of the research revolving around stomatal responses and their role in controlling leaf hydration  
66 use measurements of stomatal responses and transpiration by leaf gas exchange at a sufficient time  
67 resolution and accuracy. Unfortunately, the property to be controlled, namely LWC, cannot easily be  
68 measured simultaneously with equally high accuracy and temporal resolution which impedes the  
69 analysis of the dynamics of feedback control. The classical approach to measure water potential in a  
70 pressure bomb (Scholander et al. 1965) is destructive and non-continuous. However, several  
71 nondestructive methods to sample LWC or other related measures (turgor, water potential, sap flow)  
72 have been developed (Zimmermann et al. 2008; Martinez et al. 2011; Davis et al. 2012; Defraeye et al.  
73 2014; Dadshani et al. 2015; Baldacci et al. 2017; Fariñas et al. 2019). Each of these methods has its  
74 experimental benefits, but also comes with specific drawbacks. Some measurements have a poor time-  
75 resolution (sap flow recording), affect water status by impeding transpiration and/or changing the  
76 energy balance (e.g. psychrometric methods and those methods requiring continuous clamping of  
77 leaves) or cannot easily be used within cuvettes with full control of the leaf-micro-environment. For  
78 contactless measurements of LWC reflection or absorption of acoustic or electromagnetic waves can  
79 be used. Noninvasive ultrasonic resonance spectroscopy (Sancho-Knapik et al. 2016; Fariñas et al.  
80 2019) has proven to yield signals closely related to leaf hydration in many species. While this  
81 approach is based (indirectly) on the covariation of leaf mechanic properties with water content,  
82 measuring the interaction of electromagnetic waves of different frequencies (Sancho-Knapik et al.  
83 2011; Browne et al. 2020) with water molecules promises to offer a more direct measure of water  
84 content. Leaf transmission and reflection spectra in the visible light and infrared range (Jacquemoud &  
85 Baret, 1990) have widely been employed for contactless determination of leaf and vegetation water  
86 content (Thomas *et al.*, 1971; Inoue *et al.*, 1993; Cozzolino, 2017; Braga *et al.*, 2021). This approach  
87 has gained importance especially in satellite and aerial vehicle based remote sensing. While absorption  
88 by liquid water is an important determinant of IR absorbance and reflection spectra of leaves, a  
89 straightforward derivation of LWC from spectral measurements is hindered by interfering effects of  
90 e.g. dry matter content, metabolite composition and leaf structural parameters which can widely vary  
91 between and even within species. Various solutions to this problem have been proposed and  
92 successfully applied: Inclusion of more spectral bands up to continuous spectra, development of  
93 various indices (Jiang et al. 2018; Braga et al. 2021; Li et al. 2021), inversion of leaf optical models  
94 (Jacquemoud and Baret 1990; Féret et al. 2019) and neural networks (Conejo et al. 2015; Koirala et al.

95 2020; Braga et al. 2021) successfully aid in deriving specific leaf properties in the presence of  
96 confounding variation in overlapping spectral effects.  
97 The approach presented here, however, attempts to simplify the estimation of LWC from spectral  
98 measurements by performing calibrations for individual leaves. In this way, even limited spectral  
99 information from key spectral ranges strongly influenced by liquid water could be sufficient to get  
100 precise measurements of LWC. The separation from non-water-related confounding leaf optical effects  
101 is assigned to the calibration procedure which is performed on and valid only for the measured leaf.  
102 By measuring in only two spectral IR ranges (Seelig et al. 2009) a simple and miniaturized  
103 measurement setup based on two LED's and one photodiode is possible. Using this approach a non-  
104 invasive, precise and continuous optical method to record of LWC is presented which puts little  
105 constraint on simultaneously occurring other measurements or the choice and control of experimental  
106 conditions. Example measurements demonstrate how the combination of this measurement of LWC  
107 with other methods can yield better insight into leaf water budgets and the regulatory processes behind  
108 leaf water homeostasis.

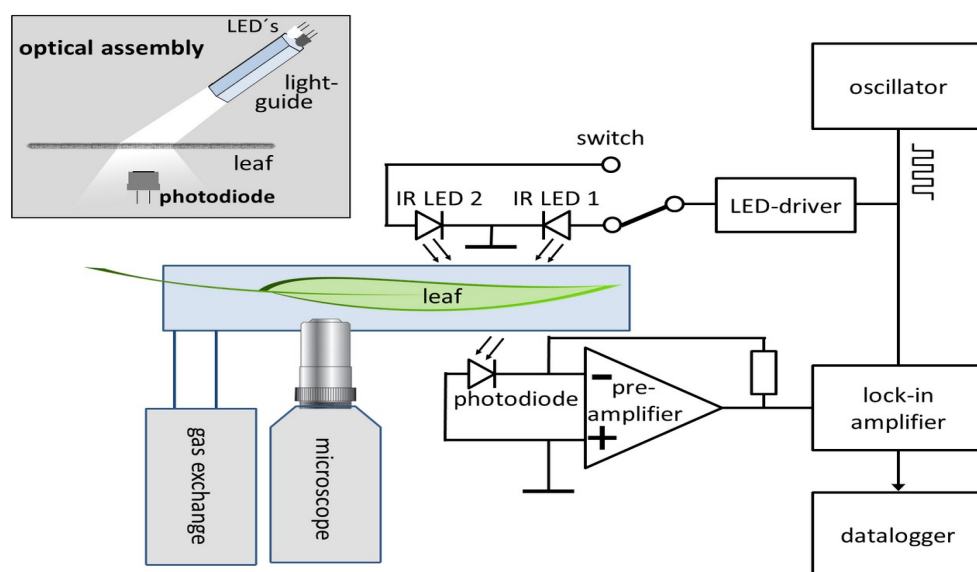
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## 110 **Materials and Method**

### 111 *Principle of the water content sensor*

112 The transmission of light through leaf tissues depends on specular reflection, internal reflection,  
113 scattering and absorption (Govaerts et al. 1996). Varying water content causes changes in the  
114 absorption spectrum especially in the distinctive absorption bands of liquid water. In the presented  
115 device, the infrared transmission at 1450 nm, a local maximum of the water absorption spectrum, is  
116 used as an indicator of LWC. Initial experiments with a first prototype using only the 1450 nm spectral  
117 band confirmed a strong response to LWC. However, the leaf clamped by an annular clamp around the  
118 measured area, moved slightly due to turgor changes which strongly influenced the IR transmission  
119 signal. These movements were evident as a changing focus position of the microscope when  
120 simultaneously observing the lower epidermis. Leaf movements change the distance between the light  
121 sensing photodiode and the leaf tissue diffusing the measurement beam and therefore strongly  
122 influence incident irradiation on the sensor surface. A firmer fixation of the leaf at the spot of  
123 measurement, however, is undesirable, as it would inhibit transpiration and thus disturb leaf water  
124 relations. This problem was overcome by using a ratiometric measurement principle with an  
125 additional beam at 1050 nm with little absorption by liquid water. The 1450 nm and 1050 nm bands  
126 were chosen, because they represent two bands which are differentially affected by leaf water content  
127 and because both spectral ranges fall into the sensitivity range of InGaAs photodiodes which allowed  
128 for a simple setup for transmission measurements basically consisting of only one photodiode and two  
129 LED's. Errors due to leaf movements should affect both measurement wavelengths similarly and be

130 canceled out in the transmission ratio. The same reasoning applies for other errors in the optical and  
131 electronical signal chain, which affect both beams independently of their wavelength.  
132 The ratiometric measurement was accomplished by measuring alternately the light transmission of the  
133 light emitted by 1450 nm and 1050 nm LED's (LED1450-03, Roithner Laser, Wien, Austria;  
134 LED1050E Thorlabs, Munich, Germany) with an InGAs photodiode (LAPD-1-09-17-TO46,  
135 Roithner Laser, Vienna, Austria). The measuring light was pulsed at 1,2 kHz and photodiode output  
136 amplified phase-sensitive with a lock-in-amplifier to allow measurements irrespective of ambient  
137 radiation (Fig 1). The optical assembly consists of the two LED's ( pointing to the upper leaf surface at  
138 an angle of 45° thus avoiding shading of the measured area when using perpendicular illumination.  
139 Radiant power density as estimated from manufacturer data for the LED's when driven with a current  
140 of 20mA in a 50% duty cycle and an illuminated area of 0,5 cm<sup>2</sup> is lower than 14 W/m<sup>2</sup>, which was  
141 assumed to be negligible (approx. 1.4% of full sunlight radiant density). The beams of the LED's  
142 were homogenized by a hexagonal homogenizing light pipe (#63-088, Edmund Optics, Barrington,  
143 U.S.) to ensure identical and homogenous illumination for both wavelengths. The photodiode placed  
144 5-10mm below the illuminated area (Fig. 1) picked up the transmitted diffuse light of the LED's.  
145



146 *Fig. 1*  
147 *Measuring principle, simplified schematic circuit and optical assembly of the device for the measure-*  
148 *ment of leaf water content integrated into a cuvette system with simultaneous microscopical observa-*  
149 *tion of stomata.*

150

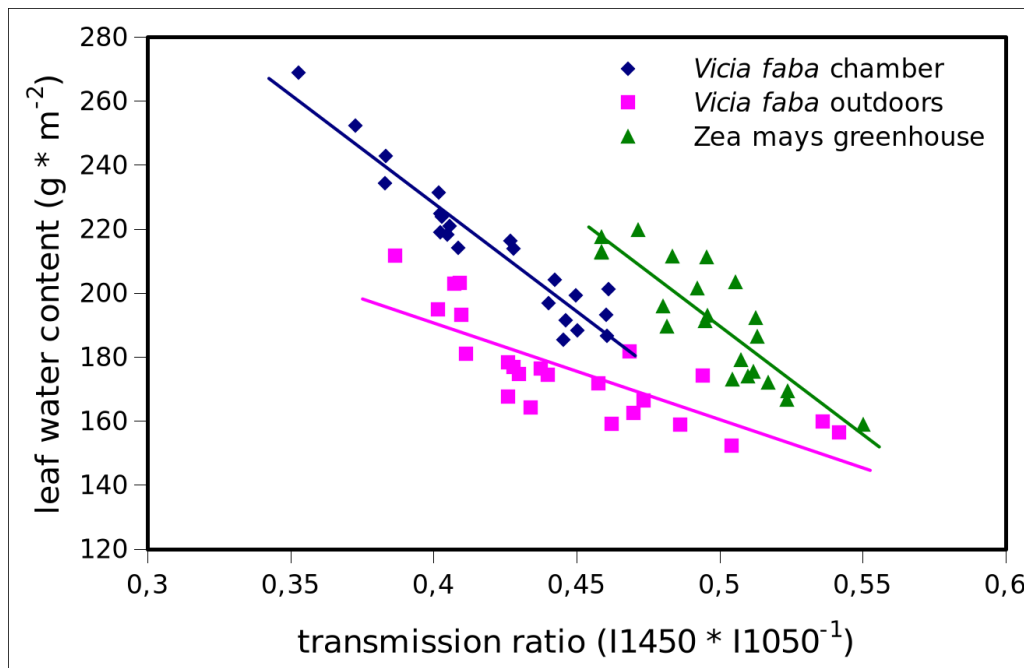
151 In order to standardize the I1450/I1050 ratio signal across measurements, the raw signals were divided  
152 by the signal obtained using a wavelength independent diffusive standard made of a matte glass slide  
153 with scattering properties similar to a typical leaf. This adjustment procedure is performed every time  
154 the measurement setup has changed (e.g. changed distance, angle, LED-currents) and warrants  
155 comparable I1450/I1050 ratios between measurements.

156

157 Calibration

158 Accurate calculation of LWC from adjusted I1450/I1050 requires a calibration which is dependent on  
159 among others leaf structural parameters and therefore specific for each observed leaf or at least  
160 specific for the observed species and growing condition. Calibration can be performed in three  
161 different ways. Up to now calibrations mostly were performed after the experiment specific for each  
162 measured leaf by simultaneously recording LWC with an independent method and I1450/I1050 ratio.  
163 This can be done either by detaching the leaf and weighing the entire leaf clamp together with the leaf  
164 on a scale while continuing to measure IR-transmission, which of course requires precautions to keep  
165 the force exerted by the cable constant. Another way to calibrate is only possible if the sensor is  
166 installed in a gas exchange chamber for continuous transpiration measurements: After cutting the  
167 petiole with the leaf still enclosed in the cuvette I1450/I1050 ratio and transpiration are measured  
168 simultaneously for about 0.5-1 hours. The accumulated transpirational water loss for each measured  
169 infrared transmission ratio during this period is finally added to the final LWC determined by  
170 weighing fresh leaf weight at the end of the leaf drying period in the cuvette and dry weight after  
171 complete drying in an oven. As most of the experiments up to now have been performed in a cuvette,  
172 this procedure was the usual way to obtain an accurate per leaf calibration.

173



175 Fig. 2

176 Calibrations of the leaf water content sensor for leaves of *Vicia faba* grown hydroponically in climate  
177 chamber, *Vicia faba* grown outdoors in Mitscherlich-pots and *Zea mays* plants grown in a greenhouse.  
178 On 22 detached leaves of each species resp. treatment leaf water content was measured gravimetri-  
179 cally simultaneously with the transmission ratio I1450/I1050. To induce variation in LWC, 11 of the  
180 leaves were measured immediately after cutting in the fully turgescient state and 11 after c. 10 minutes  
181 of passive drying under c. 22°C and 50% rH.

182

183 Another method of calibration is applicable when per leaf calibration by the balance method and the  
184 transpiration based method are not possible. Regression on accumulated data of several leaves of a  
185 species or variety under defined growing concitions can result in a calibration specific for a batch of  
186 plants in an experiment (fig. 2). To demonstrate such calibrations and their associated measurement  
187 uncertainties, leaves of *Vicia faba* plants from two growing conditions (outdoors in pots or  
188 hydroponically in a climate chamber) and of *Zea mays* grown in pots in a greenhouse were detached in  
189 the fully turgid state, and either immediately weighed and IR transmission ratio measured with the  
190 LWC sensor or left to dry under room conditions for c. 10 min before measurement. The obtained  
191 relationships between the IR transmission ratio followed a linear relationships as long as LWC was  
192 above the wilting point. As the sensor was only used within this range a linear calibration was  
193 sufficient and could be used to calculate leaf water contents from IR transmission ratios. The  
194 calibration lines differed in slope and offset between species and growing conditions, showing that not

195 only each species requires an individual calibration, but also that growing conditions can substantially  
196 change the relation between the IR transmisson ratio and LWC. After calibration for a certain variety  
197 under the experimental growing conditions, a measurement uncertainty (experimental standard  
198 deviation) of between 6 to 9 g m<sup>-2</sup> was achieved, which is between 3 to 6% of the total leaf water  
199 content.

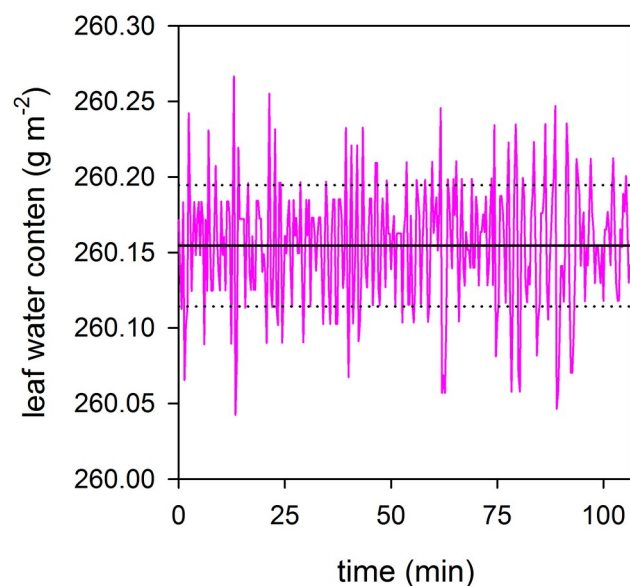
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#### 201 *Quality of the measurement*

202 One possible drawback of the ratiometric approach is the multiplication of noise from the two  
203 channels. The final signal-to-noise ratio, however, remains fully sufficient (Fig 3) with an error margin  
204 of  $\pm 0.0155$  g\*m<sup>-2</sup> when using oversampling and averaging to a 20s interval. To put this into perspective  
205 this signal noise amounts to about 0,04% of the amplitude of changes in LWC observed during a  
206 drought experiment leading to non lethal wilting (fig. 7). This high precision together with the  
207 continuous nature of this measurement allowed to discriminate changes in LWC after a change in  
208 conditions already after 20 to 30 seconds (figs 4-6).

209

210 *Fig. 3*



211 *Demonstration of signal to noise ratio (SNR). The measurement was performed on an attached leaf of*  
212 *Vicia faba during a period of stable leaf water content with a running average applied over 30 sec.*  
213 *Dotted lines indicate  $\pm$ standard deviation.*

214

#### 215 *Experimental setup*

216 The experiments presented here serve to demonstrate sensor performance and possible applications in  
217 combination with other methods, They were conducted with the LWC-sensor installed in a cuvette of a



218 customized gas-exchange system (Walz GmbH, Effeltrich, Germany) allowing LWC measurements  
219 under controlled temperature light and VPD simultaneously with H<sub>2</sub>O gas-exchange measurements  
220 and microscopic observations of stomata on the abaxial leaf surface of the same leaf see (Kaiser and  
221 Legner 2007). This not only enables the transpiration based calibration procedure described above but  
222 also allows to calculate a continuous leaf water budget from measured transpiration and rate of change  
223 in leaf water content. The rate of influx through the petiole is the difference between efflux by  
224 transpiration and the rate of change in LWC.

225

226 
$$\text{Influx} = \frac{\Delta \text{LWC}}{\Delta t} + E \quad (\text{mmol} \cdot \text{m}^{-2} \cdot \text{s}^{-1})$$
 with Influx = petiole water influx, E = transpiration

227 rate

228

229 Hereby, a continuous determination of leaf water fluxes is possible.

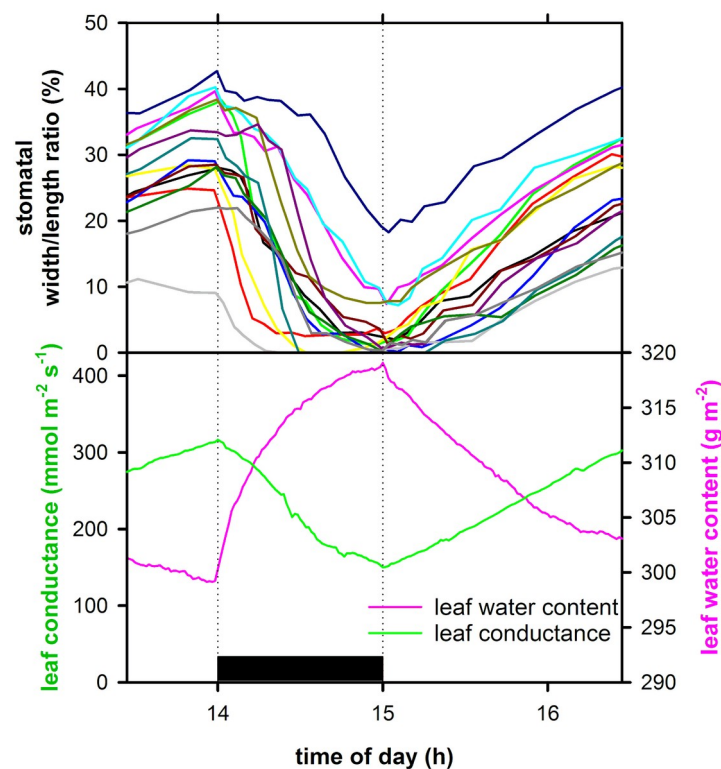
230 The sensor was tested on leaves of *Vicia faba* L. grown in a greenhouse under ample supply of light  
231 water and nutrients. Variations in LWC were induced by excision of the leaf, darkening, changing  
232 VPD and gradual soil drying followed by sudden rewatering.

233

## 234 **Results and discussion**

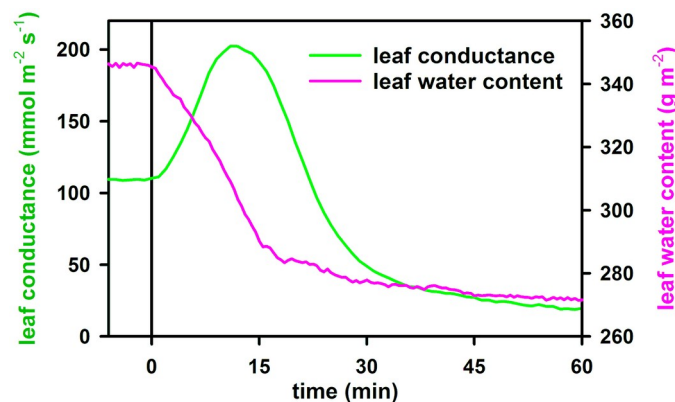
235 The sensor uses the IR-absorbtion ratio at 1450 nm vs 1050 nm to provide a precise real time  
236 measurement leaf water content. This ratiometric dual-beam transmission measurement using one  
237 water sensitive and one less water sensitive has been successfully applied previously (Sancho-Knapik  
238 et al. 2011; Cecilia et al. 2022) to record variations in leaf water status. The goal of the approach  
239 presented here was to overcome the problems of using only a limited spectral information on  
240 structurally complex and diverse objects with different biochemical compositions to estimate the  
241 content of water which is only one of several effector of changes in IR transmission. While the  
242 transmission ratio at the spectral bands around 1450 and 1050 nm has a strong relation to LWC  
243 content, the interfering influences of leaf structure and differences in biochemical composition  
244 preclude the possibility of a universal calibration valid across species and leaf morphologies. This is  
245 evident from the calibrations for maize and faba bean (fig. 2). The different calibration parameters for  
246 different growing conditions demonstrate that a calibration per species may not be sufficient, but that  
247 plants from different growing conditions require a separate calibration. The reasons for differences  
248 between calibrations are speculative. In the case of the *Vicia faba*, a 25% higher dry mass was found in  
249 the outdoor grown leaves, which might explain the differences, but they may also result from  
250 variations in internal leaf structure or reflective properties of the leaf surface. Irrespective of the  
251 reasons for variations in calibration parameters, the examples (fig. 2) demonstrate that a calibration for

252 a batch of experimental plants from identical growing conditions can result in an accuracy of LWC  
253 estimation of c. 3-6%. In many experiments this accuracy can be sufficient especially if the focus is  
254 on detecting relative changes of LWC where precision is more important than accuracy. In the  
255 experiments presented here however, the simultaneous measurement of transpiration offered the  
256 opportunity to calibrate the sensor specifically for the leaf under investigation using cumulated  
257 transpirational water loss of the detached leaf together with gravimetric determination of the  
258 remaining LWC to achieve a both accurate and precise calibration. Calibrated in this way, the limited  
259 spectral information from a two-wavelength-measurement is sufficient to record water contents both  
260 precisely and accurately. It should be kept in mind, however, that the spot of LWC measurement is  
261 small and may not be fully representative for the whole leaf whose water content is determined by the  
262 integrating weight and transpiration measurements during calibration. Future technical improvement  
263 should address this problem by using more or wider measurement beams.  
264  
265



266 *Fig. 4*  
267 *The typical dynamic response of leaf conductance, leaf water content and apertures of 15*  
268 *microscopically observed stomata to intermittent darkening (1h). The experiment was performed at a*  
269 *temperature 24°C, a VPD of 1.05 kPa and a PPFD when illuminated of 450  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .*  
270 *Examples of applications*

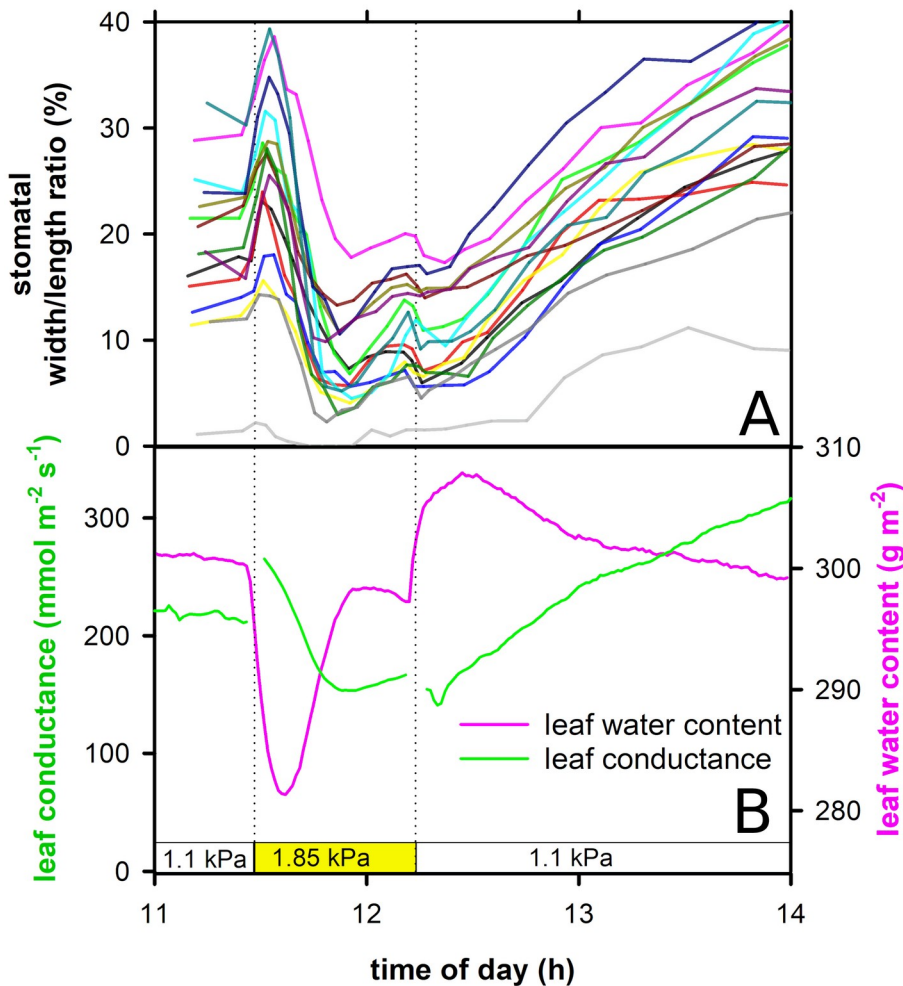
271 In order to test the usability of the sensor to detect fluctuations in LWC induced by changes in  
272 environmental conditions in different experimental settings, the sensor was installed into a gas-  
273 exchange cuvette with full control of temperature, VPD and Light and the option to measure  
274 transpiration and stomatal apertures simultaneously (Kaiser 2009). Fluctuations of LWC were induced  
275 by varying PPFD, VPD, cutting the petiole and by varying the soil water content.  
276 Darkening a transpiring leaf will lead to a decrease of transpiration due to a quick drop in leaf  
277 temperature and subsequent stomatal closure. The changed flow balance between uptake through the  
278 petiole and transpiration will lead to an increase in LWC. The dynamics of this response was observed  
279 in detail with the LWC sensor (fig. 4) in combination with cuvette measurements of leaf conductance.  
280 Upon darkening LWC content increased by about 7%, approaching a new equilibrium after 1h. After  
281 re-illumination this process was reversed. The LWC sensor tracked these changes in high temporal and  
282 signal resolution which showed important details. For example, the change of LWC followed a typical  
283 relaxation kinetics with the rate of change being largest just after darkening/re-illumination and  
284 decreasing towards the new equilibrium. Together with the simultaneously measured stomatal  
285 responses of stomata and gas fluxes a dynamic in-situ analysis of leaf water relations is possible.  
286  
287



288 *Fig. 5*  
289 *Response of LWC and leaf conductance to detachment of the leaf from the stem. The experiment was*  
290 *performed at a temperature of 24°C, a VPD of 1.05 kPa and a PPFD of 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Representa-*  
291 *tive response of numerous recordings on detached leaves.*

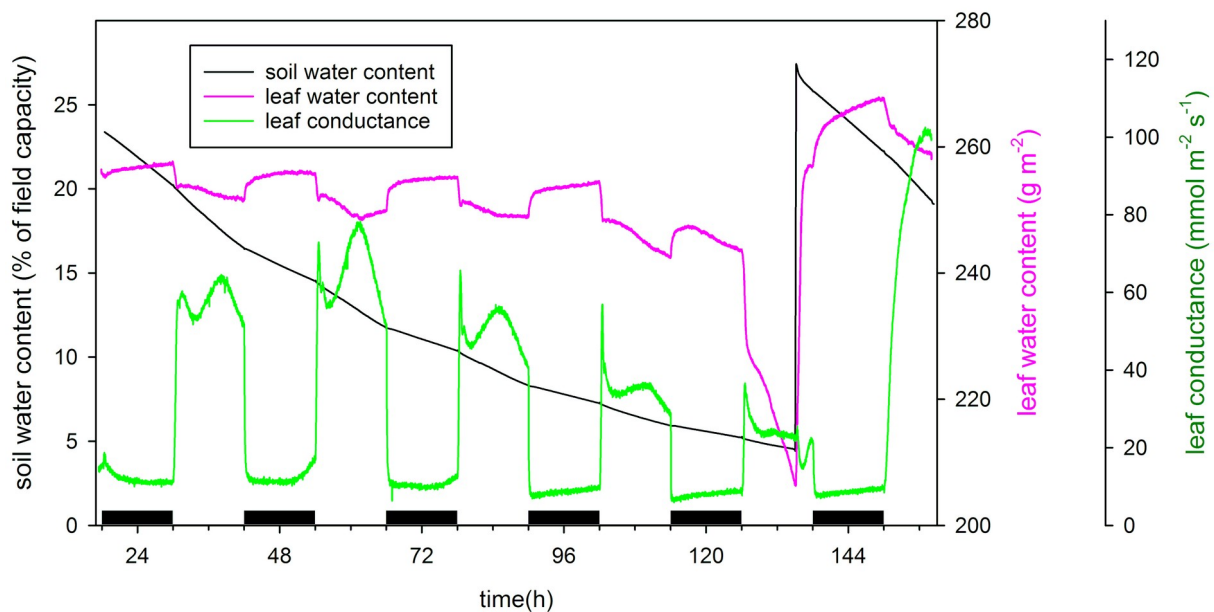
292  
293 In another experiment the response to cutting a leaf was measured (fig. 5), demonstrating the classical  
294 Iwanoff-effect (Iwanoff 1928) of hydropassively opening of stomata upon the onset of water loss. This  
295 temporal opening is known to rely on the so called mechanical advantage of guard cells (De Michele  
296 and Sharpe 1973) which causes opening of stomatal pores after a turgor decline due to a negative

297 water balance . In this experiment the LWC-sensor informed about the dynamics of leaf water content,  
298 showing that the maximum rate of water loss coincided with the maximum of leaf conductance 15  
299 minutes after cutting the petiole. This demonstrates the action of a positive feedback-loop between  
300 ‘wrong way’ stomatal responses and leaf hydration (Cowan 1972) which initially led to an  
301 acceleration of water loss until active stomatal closure after 20 min was able to counteract and to close  
302 stomata.  
303



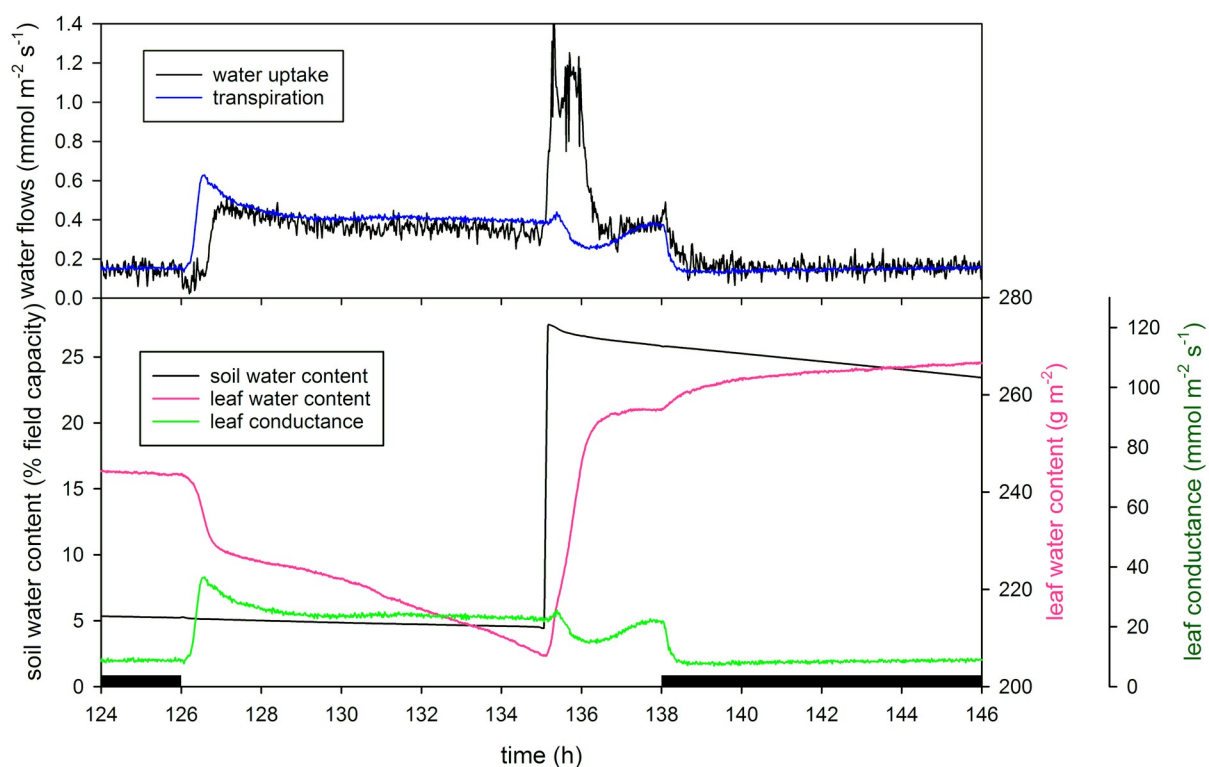
304 Fig. 6  
305 Typical responses of stomata, leaf conductance and LWC to a temporary increase (1h) of VPD from  
306 1,1 kPa to 1,85 kPa. A) Microscopically observed apertures of 15 individual stomata. B) LWC and leaf  
307 conductance. The measurement of  $g_L$  was interrupted during transitions to a different VPD. The exper-  
308 iment was performed at a temperature of 25°C, and a PPFD of  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ .  
309  
310

311 The measurement of a LWC response to a more temporal disturbance of leaf water status by a  
312 temporal increase of VPD from 1.1 kPa to 1.85 kPa (fig. 6) revealed a complex dynamic of LWC and  
313 its interaction with stomatal movements and gas exchange. After switching to high VPD, similar to the  
314 previous leaf cutting experiment, a temporal decline in LWC caused temporary hydropassive stomatal  
315 opening followed by active closure and consequently recovery of LWC to a value almost as high as in  
316 lower VPD. Switching back to low VPD revealed the inverse dynamic, an increase in LWC caused  
317 transient further stomatal closure. At this time, the positive feedback between leaf hydration, passive  
318 stomatal responses and transpiration supposedly lead to leaf water contents much higher than at the  
319 beginning. A delayed active reopening by increased transpiration restores the previous balance  
320 between water uptake into the leaf and transpiration and a recovery of the initial LWC. Notably, while  
321 short term LWC disturbances apparently were accentuated by hydraulic positive feedback, longer term  
322 stomatal adjustments recovered a similar LWC irrespective of VPD as would be expected from a  
323 feedback control of LWC. This suggests that the inclusion of LWC measurements into such  
324 experiments can provide the missing causal link between transpiration induced changes in leaf tissue  
325 hydration and stomatal feedback responses and could allow a more complete analysis of the  
326 interactions of hydraulic and pyhsiologic processes and their dynamic interaction.  
327



328 *Fig. 7*  
329 *Leaf water content and leaf conductance during a soil drying and rewatering pot experiment on a leaf*  
330 *enclosed in a gas exchange chamber. Soil water content was measured by placing the pot on top of a*  
331 *continuously recording balance. The leaf was kept at 20°C, a VPD of 1.5 kPa and a 12/12 L/D cycle*  
332 *with a PPFD of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .*

333 The method combination of gas-exchange with LWC-measurement was also used in a long term soil  
334 drying and rewatering experiment (fig. 7 and 8) , to assess whether the sensor provides stable and  
335 meaningful signals over terms of several days. During gradual decline in soil water content, LWC  
336 stayed largely constant for the first four days and only then started to decline. Stomatal conductance  
337 however started to decline earlier and was clearly reduced on the last two days of the experiment apart  
338 from a short temporary increase in the morning. Apparently stomatal responses at first successfully  
339 acted to preserve water. As LWC at this stage was not yet affected, this closure can be seen as ‘pre-  
340 emptive’. Only on day five LWC started to decrease at an increasing rate leading to a quick and  
341 visually apparent wilting process. At this point, by rewatering the plant, a recovery was initiated and  
342 monitored.



343 Fig. 8

344 Leaf water content, leaf conductance and balance of water flows during wilting and re-watering of a  
345 leaf. Detail from the final stage of the soil drying experiment shown in fig. 7. The upper panel shows  
346 measured transpiration and water uptake through the petiole as calculated from the leaf water balance  
347 equation.

348

349 The dynamics during the final stage of wilting and the subsequent recovery here serve as an example,  
350 how a combination of LWC and transpiration measurements can be used to calculate water uptake  
351 through the petiole, thereby making up a continuous leaf water balance (fig 8). The comparison of leaf



352 water uptake and transpiration showed that starting from the morning of the last day of the drought  
353 experiment, transpiration exceeded uptake, resulting in steady decline of LWC until wilting was  
354 reached. Rewatering after observation of visible wilting resulted in a rapid recovery completed within  
355 less than an hour. Noteworthy again is, that continuous observation of LWC and transpiration allows a  
356 flow rate determination even in these rapid transitory events which are governed by rapidly changing  
357 flow rates and leaf capacitance (Blackman and Brodribb 2011; Schymanski et al. 2013). Strikingly,  
358 LWC after recovery was higher than at the beginning of the experiment, which could be explained by  
359 osmotic adjustment during the 5 days of gradual drying resulting in increased osmotic potential and  
360 hence higher final water content. In *Vicia faba*, changes in ion fluxes (Shabala et al. 2000) and in  
361 expression patterns of sugar metabolism related genes conforming to processes of osmotic adjustment  
362 (Ghouili et al. 2021) were found after exposure to osmotic stress. The reports on the actual existence  
363 and magnitude of osmotic adjustment, however, remain contradictory (Amede et al. 1999; Khallafallah  
364 et al. 2008; Abid et al. 2017). The LWC sensor in combination with other direct measurements of  
365 osmotic or total water potential appears as a promising tool to investigate the research field of osmotic  
366 adjustment.

367

368 To summarize the lessons learned from these example experiments, the presented method appears best  
369 suited for experiments requiring precise continuous recording of relative changes in LWC with  
370 sufficient SNR to detect even slight variations in near real time. The accuracy depends on the chosen  
371 calibration method and is high for a single leaf calibration, whereas the less accurate species specific  
372 calibration is sufficient for many applications and will maintain the high precision when measuring  
373 relative changes in LWC. The use of only two wavelengths thus means, that in order to achieve both  
374 high precision and accuracy the additional effort of a per leaf calibration procedures is required. The  
375 reward is however a small and simple sensor setup which does not impose many limitations on the  
376 choice of other simultaneous measurements. Here, combinations with cuvette based gas exchange  
377 measurements under control of gas composition, and light level together with in-situ microscopy are  
378 demonstrated. Combinations with other non-invasive optical methods like chlorophyll fluorescence  
379 probing and spectroscopic measurements are feasible.

380

381 In combination with recording of leaf gas exchange a real time balance of leaf water uptake and loss  
382 via transpiration can be calculated enabling e.g. an analysis of leaf water capacitances and flow  
383 resistances (Blackman and Brodribb 2011).

384 It might be questioned that LWC is a relevant parameter for leaf water relations at all. The significance  
385 of measures for water status has been under discussion (Jones 2007). Water potential, osmotic  
386 potential and turgor potential are linked and co-varying according to the water potential equation with

387 changing leaf water content. Frequently, water potential is considered as the most important measure,  
388 as it ultimately determines the direction of water transport. For many physiological processes however,  
389 leaf water content appears to be a better determinant (Sinclair and Ludlow 1985). For example, LWC  
390 decline has been linked to inhibited Photosynthesis, (Kaiser 1987; Lawlor and Tezara 2009) and  
391 altered Chloroplast movement (Nauš et al. 2016). It should be considered here, that small variations in  
392 LWC typical for sub-stress fluctuations in water status will only have a relatively small impact on  
393 water potential, while directly affecting cell volume and turgor pressure. Changes in cell volume and  
394 turgor pressure are controversially discussed as properties being sensed by cellular mechanisms and  
395 feeding back into processes regulation water status (e.g. activation of proton pumps, adjustment of  
396 osmotic potential, guard cell responses, activation of key enzymes of ABA synthesis (McAdam and  
397 Brodribb 2016; Sack et al. 2018; Zhang et al. 2018). If changes in cell volume are the relevant  
398 property, direct measurements of LWC are a useful experimental tool. Continuous non-invasive turgor  
399 measurements would also be desirable but they turned out to be difficult and attempts to use  
400 mechanical force sensors (Zimmermann *et al.*, 2008) while providing continuous recordings of relative  
401 changes suffer from lack of absolute calibration and disrupt the local tissue water balance by blocking  
402 transpiration at the measured site. Instead of direct turgor measurements, water content can serve as a  
403 good proxy for turgor as long as cell volume and cell wall elasticity remain constant. Although drought  
404 can induce long term changes in cell wall elasticity (Martínez *et al.*, 2007) over short term cell wall  
405 properties can be assumed to be constant resulting in a fairly constant turgor-volume relation which  
406 makes LWC a good proxy for turgor changes. Measurements of LWC are definitely meaningful when  
407 performed simultaneously with transpiration measurements, allowing a quantitative coverage of all  
408 liquid and gaseous water exchanges of a leaf.

409

#### 410 **Acknowledgements**

411 I gratefully acknowledge the skillful technical assistance of late Frank-Peter Rapp. Thanks go to Jon  
412 Henningsen for aid in calibrations.

413

#### 414 **Data availability statement**

415 Data are available on request from the author.

416

#### 417 **References**

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