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Publication Date

2022

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Efflux Quantification and the Evaluation of different Pressurization Rates and Regimes in Pressure Chamber Measurements

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Horticulture & Agronomy

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

i

Abstract

The pressure chamber method has been widely used for decades to measure water potential in plant tissues. In this method, air pressure around the tissue being tested is increased at a constant rate, and the pressure required to visually observe the initiation of sap efflux from the tissue (the endpoint pressure) is used as the measure of the tissue's water potential. We developed a novel system for quantitatively measuring the volume of sap efflux over time, and hence for more objectively determining the endpoint pressure. In this system, sap is absorbed through a membrane under hydrostatic tension and the sap volume is measured directly. We used the system to test the influence of different pressurization rates and regimes on pressure chamber measurements of water potential in greenhouse-grown grape leaves. We found that increasing pressurization rates resulted in progressively lower apparent water potential compared to a control (slow) pressurization rate. While the underlying causes are not entirely clear, a time lag for sap flow to be detected appears to be the likely cause. Increases in temperature were also associated with increases in pressurization rates, and such temperature effects have been suggested in the literature as causing real (not apparent) changes in water potential. However, direct measurement of leaf temperatures inside the pressure chamber did not support this hypothesis. The application of split pressurization regimes however, where a high initial rate is followed by a slow rate for endpoint determination, showed consistent endpoints, essentially the same as the control method, regardless of the initial rates used, and hence these can be used to accomplish both accuracy as well as time efficiency in pressure chamber measurements. Further investigation and data from more species will be needed to test the generality of these conclusions.

Table of Contents

iv

List of Figures

List of Tables

Introduction

Almost 60 years ago Scholander et al. (1964) described the pressure chamber to measure the tension in the xylem sap of leafy shoots. This was the start of the pressure chamber's success story and its wide use in the field of plant water relations – e. g. Shackel (2011). Generally, there are two different forms of application for the device: First, the determination of water potential in plants (e.g., Boyer (1967) and Milliron et al. (2018)) and secondly, the purposeful extraction of sap from plant samples, in order to manipulate their water status or to evaluate their hydraulic properties (Tyree et al. 1974; Tyree and Dainty 1973; Zwieniecki et al. 2007). The setup of the pressure chamber is simple: Plant material gets enclosed and sealed in the chamber except from a small portion, which protrudes into the atmosphere (Scholander et al. 1965).

The theory of the pressure chamber assumes an equilibrium in water potential within the sample (Scholander et al. 1964; Tyree and Hammel 1972). This means that the water potential is the same throughout the sample and does not change. Given such equilibrium, there should be a certain pressure inside the chamber that exactly balances the tension in the apoplast's sap of the sample (Tyree and Hammel 1972; Scholander et al. 1965). Any pressure increase on top of this balancing pressure should lead to sap flowing out of the protruding end (Tyree and Hammel 1972), but at the balancing pressure itself, no fluid outflow should occur (Tyree and Hammel 1972). An additional assumption allows the measurement of water potential with the pressure chamber: When assuming that the apoplastic sap has a negligible amount of solutes – which often appears reasonable (e.g., Duniway (1971b) and Tyree and Hammel (1972)) – then solely the tension in the apoplast determines the water potential of the sample (Ritchie and Hinckley 1975).

The reading of the pressure chamber – often called "endpoint" (Baughn and Tanner 1976; Turner 1988) – is the representation of the pressure inside the chamber that balances the tension in the sample's apoplast (Ritchie and Hinckley 1975). For the endpoint's indication, the end of the sample's protruding

part is visually observed by the user (Scholander et al. 1964). Surprisingly, people have applied a whole variety of criteria for the determination of the endpoint – for example: "sap reappeared" (Baughn and Tanner 1976), "beginning of water exudation" (Milliron et al. 2018), "menisci appeared" (Potvin and Werner 1984), "sap just returns" (Turner 1981), "water should appear" (Ritchie and Hinckley 1975), "liquid just wets the surface" (Scholander et al. 1965), or "return [of] the meniscus of the xylem sap to the cut surface" (Boyer 1967). These descriptions appear to be distinct, which would be a major issue considering all criteria aimed to serve the same purpose: The accurate determination of the specific pressure that exactly balances the tension in the apoplast – the accurate determination of the endpoint.

It is astonishing that a measurement device like the pressure chamber, which is used for decades and of such importance, appears to have no universal criterion for when its measurement shall be taken. In the following we therefore want to present a thought experiment which aims to derive such a universal criterion. An applicable criterion needs to be visually detectable by the user, since visual observation of the protruding end is how the device is generally run. We know that given the perfect scenario of equal water potential throughout the sample, the endpoint should ideally be indicated at the pressure inside the chamber that exactly balances the tension in the apoplast. But what happens at this pressure at the protruding cut of the sample that can be visually detected by the user? We know from experiments by Wei et al. (2000) that the tension within the xylem decreases 1:1 with the pressure increase within the chamber. But this should lead to nothing more pronounced than changes (increases) in the radius of curvature at the tiny surfaces of the sap menisci within the xylem vessels (Tyree and Hammel 1972). Visually observing such alterations appears practically impossible. But is there anything more noticeably happening when the chamber's pressure finally balances the tension in the apoplast exactly? We assume not: All that should have changed is that the sap menisci within the xylem vessels now have a radius of curvature that is infinitely large, meaning that the surface of the menisci is almost flat, but like with the curved menisci – it wouldn't be possible to visually detect that. However, there should be a noticeable

2

change as soon as the pressure within the chamber increases further, because then, instead of a further decrease of the sap's tension (the tension is already balanced completely), there would be the initiation of a positive pressure. Such positive pressure in the sap should cause it to flow out into the atmosphere. And fluid occurrence at the protruding end due to efflux, could indeed be visually detected by the user. Putting these steps together and considering the happenings during a real pressure chamber measurement (where pressure is constantly rising), we surmise that during the pressure increase inside the chamber the tension in the apoplast is decreasing up to a point where there is an exact balance between the sap's tension and the chamber's pressure. However, the balancing pressure and any lower pressures shouldn't cause any visually observable alterations for the user. This ought to change abruptly since the constant pressure increase inside the chamber surpasses the balancing pressure as soon as it reached it and the subsequently caused efflux should be detectable by the user at the end of the excluded part. Balancing pressure appears to be a transient state when the pressure in the chamber is increased constantly. So, for the indication of the endpoint there would be only efflux caused by pressures that exceeded the balancing pressure, which the user could harness. We are aware of the fact that sometimes fluid occurs at the cut end before balancing pressure has been surpassed (McCown and Wall 1979; Phillips 1981). In these cases though, it is not reported that a higher pressure inside the chamber will lead to even more outflow of fluid. This is an important aspect for the differentiation from efflux caused by pressures exceeding the balancing pressure: Efflux caused by pressures that surpassed balancing pressure should increase with rising pressures inside the chamber because of the accompanied increase in positive pressure of the sap, which should propel sap outflow. Hence, as the universal criterion for endpoint recognition we want to propose: The endpoint should be taken at the first occurrence of efflux at the protruding end which is followed by even more outflow with increasing pressures inside the chamber.

Why are we so interested in the endpoint? The reason is, that even though the pressure chamber has been used for decades, there are still a number of uncertainties about its correct application. Examples

would be appropriate pressurization heights and regimes, the influence of temperature as wells as the impact of consecutive measurements. These are factors we want to consider more closely in this paper. Additionally, the error due to recutting of samples (Ritchie and Hinckley 1975; Levin 2019), the number of xylem vessels that have to show efflux (Potvin and Werner 1984), the tightness of the chamber's seal (Cheung et al. 1975; Yang et al. 2016), or the error due to excluded tissue outside the chamber (Klepper and Ceccato 1969; Tyree et al. 1978) cause tentativeness among users. Before we consider the uncertainties more closely, we want to think about how these uncertainties can be challenged: When using the pressure chamber, we want to determine the pressure at which the endpoint occurs. So, the investigation of the uncertainties' impact on the endpoint appears to be a reasonable approach. Having the user indicating the endpoint visually, involves the subjective judgement of the incident (efflux) that triggers the indication. Even though the caused variation might be small, an objective determination would still be favorable. The literature presents three different approaches that could potentially be deployed for an objective endpoint indication: One is the measurement of efflux, which – as proposed in our criterion for endpoint indication – can be utilized to determine the endpoint. Efflux measurements from pressurized plant material have been done frequently in the past by weighing the outflowing sap after pressurization beyond the endpoint. Though, the purpose was not endpoint indication, it was rather the investigation of pressure-volume curves (Tyree and Hammel 1972) or leaf hydraulics (Zwieniecki et al. 2007). It must be noted that these experiments already started at the endpoint and then applied a pressure step on top. To the best of our knowledge no efflux data exists over the whole course of a usual pressure chamber measurement, starting at atmospheric pressure and ending at the signs of sap outflow. The second way to objectively determine an endpoint, that is described in the literature, uses electrodes to measure changes in electric conductivity at the cut end due to the emergence of sap (Richter and Rottenburg 1971). This method discriminates between the two states "no efflux" and "efflux", but a quantification is not possible. Lastly, the third way is the direct measurement of water potential, at the

protruding portion of the sample, as done by Dixon and Tyree (1984) using a hygrometer – but how can we use the measure of water potential to indicate the endpoint? The theoretically ideal pressure to indicate the endpoint would be the value that exactly balances the tension in the apoplast. Under the assumption that the amount of solutes in the apoplastic sap is negligible, the water potential of the protruding portion would be 0MPa at the endpoint: The absent effect of solutes would be added the absent effect of pressure since the tension in the apoplast is exactly balanced. Even though the usage of a hygrometer at the cut end is presumably a very precise technique, the time necessary for its measurements must be considered: Dixon and Tyree (1984) reported a required time span of 1.5 to 4 minutes to reach equilibrium between the chamber's pressure and the indicated water potential by the hygrometer. The accompanied half times were 15 to 40 seconds. These features of the hygrometer's application seem problematic for our purpose of endpoint indication, since the constantly rising pressure within the chamber would lead to a time lag between the hygrometer's indications and the actual pressures in the chamber. However, the actual time lags for different pressurization rates needed to be tested to draw a sound conclusion for the hygrometer's applicability in endpoint indication during a constant pressure increase. Moreover, the quantification of efflux is not possible using a hygrometer. So, literature presents possible ways for objective endpoint indication, but the quantification of efflux over the whole course of a pressure chamber measurement and thereby the indication of the endpoint, has not yet been done.

Our interest in the endpoint was propelled by the existence of a number of uncertainties around the chamber's usage. Temperature is one of these factors causing uncertainty, since temperature changes are presumably accompanied with changes in water potential (Puritch and Turner 1973; Ritchie and Hinckley 1975; Tyree et al. 1974). This is an important aspect since the pressure alterations inside the chamber are accompanied with temperature changes (Puritch and Turner 1973). There is agreement that the osmotic potential, as one component of total water potential, probably depends on temperature

according to the Van't Hoff's principle (Puritch and Turner 1973; Ritchie and Hinckley 1975; Tyree et al. 1974): An increase in temperature provokes a more negative osmotic potential. However, when Tyree et al. (1974) actually tested the influence of temperature on total water potential on Hemlock shoots (Tsuga Canadensis), they observed that only samples at low water potentials (<-2.4MPa) acted according to the Van't Hoff's principle. Samples at higher water potentials showed less of a decrease with increasing temperatures than the principle suggests. Moreover, at water potentials higher than about -1.8MPa the authors found that the direction of the temperature effect had changed and now an increase in temperature seems to be accompanied with an increase in water potential. This led the authors to the assumption that there might be a temperature influence on two different components of water potential: The osmotic potential as well as the pressure potential. They followed, that when the cells have lost all their turgor and the pressure potential becomes zero, the water potential should be only influenced by the osmotic potential and hence the accordance with the Van't Hoff's principle in desiccated samples. When there is turgor however, the pressure potential comes into play as well. They concluded that the pressure potential's temperature dependence needed to be not only opposite but also stronger than the one of the osmotic potential – thus, the eventual shift in direction of the temperature effect in more hydrated samples. However, the authors can't explain the causes for the temperature dependence of the pressure potential: They reason that neither the thermal expansion of pure water nor of a solute solution was high enough to provoke the observed magnitude of temperature dependence. They suggest that therefore cell wall properties must depend on temperature but admit that the necessary direction of dependence is opposite to what has been reported in the literature. Therefore, either the authors assumption of the temperature dependence of the two different components (osmotic potential and pressure potential) is wrong, or there are unknown, different causes for the temperature dependence of the pressure potential. We are not aware of any other study on the temperature dependence of water potential – leaving the temperature's influence on water potential measurements understood

incompletely. To give the reader at least an impression of the problem's scale, we can note that the maximum change in water potential with temperature found by Tyree et al. (1974) was about 0.18MPa per 10°C.

What causes alterations in temperature inside the chamber in the first place? During pressurization the heat of compression raises the temperature of the gas inside the chamber. Hence, faster compression due to a higher pressurization rate should be accompanied with more heat per time within the system and thereby a faster temperature increase. Besides the influence of the pressurization rate, Puritch and Turner (1973) point at several other factors that determine the temperature change inside the device during a measurement – for example the temperature of the incoming gas or the heat exchange with the environment. The latter is influenced by the temperature difference between the inside of the chamber and the air that surrounds the device. The higher the temperature difference, the higher the heat exchange with the environment. Hence, the heat exchange with the environment counteracts the temperature increase inside the chamber due to the compression: As soon as the temperature inside the device gets higher than the temperature of the surrounding air, there will be a net heat flow from within the chamber into the surrounding environment – which constitutes a temperature reducing effect for the inside of the chamber. When Puritch and Turner (1973) measured gas temperature changes inside the device during pressurization, they expectedly reported increasing temperature rises with faster pressurization and consequently found the highest temperature increase of about 30°C for the fastest rate they applied (0.12MPa/s).

Turner (1988) reported that consecutive measurements of the same sample can lead to a slight decreases in indicated water potential. Two aspects appear reasonable to explain this observation: First, there is the sample's water loss during a measurement: Efflux at the endpoint induces water loss – at least due to evaporation at the protruding end, or the user might have even wiped it away. This loss in water should lead to a small reduction in water potential of the leaf. Hence, the subsequent pressurization

should show a little higher endpoint pressure. However, the correct increase in balancing pressure should only be detectable if water potential is the same throughout the sample before the second measurement – which brings us to the second possible cause: By finding distinct patterns of water uptake in leaf-rehydration, research suggests locally different hydraulic characteristics within the leaf (Cruiziat et al. 1980; Tyree et al. 1981; Zwieniecki et al. 2007). This could lead to the development of locally different water potentials when efflux occurs because some cells loose water quicker than others (Tyree and Hammel 1972; Tyree et al. 1973). Assuming such disequilibrium in water potential has emerged and we release the pressure after indicating the endpoint once, we can run the following thought experiment: If we take a consecutive measurement before the water potential throughout the leaf has equilibrated again, we could take an erroneous reading, since some cells – presumably the ones that lost less water during the first measurement – would be at a less negative water potential than what the equilibrated state should be and thereby leading to an endpoint at an erroneously low pressure. So, the question arises how long it takes to reach equilibrium? During his investigation in sunflower, yew and rhododendron Boyer (1967) came to the conclusion that "equilibration of the potential of water in leaf cells and xylem occurred quickly". Unfortunately, he gave no time estimates and showed no data. Later Boyer (1995) claimed equilibration of water potential would usually happen within 10 minutes, but says that in fleshy samples it might take hours or days. Again, no data is shown for these statements. However, support for the claim of a rather quick process can be found in Fulton et al. (2001): After initial alterations in indicated water potential, almost constant values over time have been found within 10 minutes after a leaf's transpiration was stopped. Under the assumption that a transpiring leaf exhibits different water potentials throughout its tissue due to resistance to water flow, changes in water potential caused by ongoing equilibration would show themselves in changes in indicated water potential. Hence, constant values over time would imply that no changes within the tissue occur anymore and thereby indicate the presence of equilibrium throughout the sample. To conclude, it appears unclear how fast equilibration happens within

plant tissue, but a rapid process seems likely. Furthermore, it shall be mentioned that besides the time for equilibration also the amount of efflux in relation to the total water content of the tissue could have an influence on how big the error in consecutive measurements could become: Ritchie and Hinckley (1975) point at a personal communication with M. T. Tyree who suggested that the volume of efflux might be so small in comparison to the total water content of the sample, that its water potential might barely be affected at all from the amount of water that is being lost during efflux. Unfortunately, no data is shown for this suggestion. So, all in all the possible error due to consecutive measurements of the same sample appears to be understood incompletely.

Generally, two ways to pressurize the chamber are being used: The application of only one rate and the application of two different rates, a split regime, where a fast rate in the beginning is followed by a slower increase once the endpoint pressure is close. The high rate in the initial part of the pressurization is what makes split regimes attractive because it presents the opportunity to shorten the time of a measurement.

Considering the continuous application of just one rate, it is surprising how little we know about the influence of the pressurization rate on the accuracy of the measurement. Generally, a slow rather than a fast rate is preferred in the literature (Ritchie and Hinckley 1975; Turner 1988; Boyer 1995), but justifications for that are sparse. Turner (1988) points at the prevention of large temperature changes – as they presumably influence water potential. Ritchie and Hinckley (1975) preferred slow rates to facilitate their endpoint indication, but unfortunately don't clearly state why this was the case. Lastly, we can find another argument favoring a slow pressurization by doing a little thought experiment: If efflux starts to occur at the cut end it will take the user a finite amount of time to process what they see at the cut, to recognize that it is efflux and to subsequently shift the line of sight towards the pressure gauge to read the current pressure. All this happens probably very fast – possibly within 1 second – but depending on the applied pressurization rate the error might be substantial. But what rates have been used to pressurize

9

the chamber? People applied a whole variety: Duniway (1971a) pressurized the chamber with less than 0.007MPa/s, Jordan (1970) used about three times of an increase (0.02-0.03MPa/s), which is only less than half the rate West and Gaff (1971) applied (0.07MPa/s) and Oechel et al. (1972) went as high as 0.1-0.2MPa/s to raise the pressure for their experiments. A reason for the big range of values might be that recommendations for adequate pressurization heights are rare and often don't rely on data. Turner (1981) suggested a rate of 0.025MPa/s when errors between 0.005MPa to 0.1MPa would be acceptable, and recommended 0.003MPa/s to 0.005MPa/s for accurate measurements. Unfortunately, no evidence is shown to support these statements. Waring and Cleary (1967) advised the usage of about 0.07MPa/s for Douglas fir trees. Additionally, they warned about the usage of too slow of a rate. However, the observed increase in endpoint pressure using slow rates may have been due to water loss via evaporation during the measurement: Their samples were not protected by any measure and water loss caused by evaporation in the chamber might lead to decreases in water potential (Boyer 1969). Unfortunately, Waring and Cleary (1967) showed no data for their claims as well. Similarly unsupported by evidence, is the suggestion of Ritchie and Hinckley (1975) to apply 0.01MPa/s. We are aware of only two studies showing actual data concerning the performance of different pressurization rates. The first is Naor and Peres (2001) who tested 5 different pressurization rates – between 0.03MPa/s and 0.25MPa/s – in deciduous tree crops. The authors took stem water potential measurements from all 5 test rates on each tree they examined. Even though not specifically stated, we assume that a new leaf was used for each pressurization. The result was the indication of decreasing water potentials with increasing rates. Dependent on the species their data showed average changes between about -0.8MPa (apple) and -1.9MPa (nectarine) per 1MPa/s of pressure increase. It shall be mentioned however that the relationship between pressurization rate and indicated water potential was not completely linear for nectarine, though our estimation for the decrease per pressurization rate is based on linearity. The authors recommended an application of 0.03MPa/s when low errors could be tolerated. For their evaluation of

the error the authors assumed that the lower the rate the more accurate the measurement becomes. Therefore, they linearly extrapolated their data to a hypothetical rate of 0MPa/s which gave an average deviation of 0.03MPa to the endpoints indicated by their slowest rate (0.03MPa/s). Under the presumption that the gain in accuracy of using a smaller rate would level off when rates get close to 0MPa/s, they figured that the true error should be smaller than the 0.03MPa suggested by the linear extrapolation. That is why they described the error as being low without giving a specific value, when applying 0.03MPa/s. It can be mentioned though, that in 3 of the 4 relations the authors show between stem water potential and pressurization rate, a linear pattern seems to exist. No explanation is given for the assumption that the effect of rate on water potential should be different when close to 0MPa/s, which makes their evaluation of the error at a rate of 0.03MPa/s appear questionable. The second study presenting actual data comes from Blum et al. (1973) where the authors gathered information about the influence of two different pressurization rates (0.033MPa/s and 0.038MPa/s). They found a slightly better performance of the faster rate, but the proximity of the rates as well as the fact that only two levels have been tested make it hard to generalize from their findings. Performance of the rates was assessed by evaluating similarity to readings from a thermocouple psychrometer and adopting the psychrometer's values as the true water potential. So, all in all, it appears surprising how little we actually know about the influence of different continuously applied pressurization rates.

The second way of pressurizing the chamber is the application of split regimes, where a fast rate in the beginning of the measurement is followed by a second, slower rate once the endpoint is close. Both the adequacy of split regimes in general as well as suitable rates for their application are not clear, as actual tests are sparse, and findings contradict each other: Similar to the continuous application of only one rate Naor and Peres (2001) are an exception in actually testing the performance of split regimes. They examined three different initial heights in 0.04MPa/s, 0.14MPa/s and 0.3MPa/s and finished the measurements in all cases by the application of 0.03MPa/s. Independent from the water status of the

sample, the rates were always switched when the pressure has reached about 1.2MPa in the chamber. The author's data showed an increase in indicated water potential with higher initial pressurization rates – about 0.6MPa per 1MPa/s. When we consider the finding for the continuous application of only one rate, that a higher rate led to a decrease in indicated water potential it appears to be a contradiction that in the application of split regimes the usage of a higher rate at least for a part of the measurement should have the opposite effect. Unfortunately, the literature doesn't present any explanation for this apparent disagreement. However, through their investigations Naor and Peres (2001) came to the conclusion that split regimes should be avoided in deciduous tree crops. The reason for the disapproval is not stated by them. In contrast to this disapproval are the findings from Hellkvist et al. (1974) who applied split regimes for their experiments in Sitka spruce. The conducted experiments did not concentrate on the adequacy of split regimes, but they noted the close agreement to readings obtained from constant pressurization at one fast rate (no value given). Additionally, they stated that the rate of pressure increase in the initial phase had no influence on the balancing pressure. Baughn and Tanner (1976) utilized split pressurization regimes in their experiments as well. Like Hellkvist et al. (1974) the authors noted in passing about their findings concerning split pressurization. They applied between 0.02MPa/s and 0.05MPa/s initially and switched to 0.005MPa/s about 0.2MPa below the expected endpoint. Five herbaceous species were measured using to following protocol: Application of the split regime, reducing the pressure slightly after the endpoint had occurred, keeping it constant for one minute and increasing the pressure slowly again until the second endpoint occurred. They found close agreement between both measurements in 4 of the 5 species. Finally, Turner (1988) wrote about the unpublished findings of M. M. Jones and N. C. Turner who reported that split regimes resulted in the measurement of lower water potentials than if one slow rate was continuously applied. Unfortunately, no specific values for the rates or deviation were given. In conclusion, we must note that there is only little knowledge about the adequacy of split regimes in general and about suitable rates for their application.

In this paper we want to address the uncertainties regarding the influence of pressurization on indicated water potential in pressure chamber measurements. The overall incomplete understanding of the topic is highlighted by the apparent contradiction concerning the impact of pressurization rate between the application of only one rate and the usage of split pressurization regimes. Consequently, we want to test the effect of different continuously applied rates as well as of split regimes on indicated water potential. Besides the effect itself we are highly interested in the possible causes for an influence of pressurization. Therefore, we try to increase the scope of our investigations by: Developing a novel method for the quantification of efflux during pressurization which will enable an objective indication of the endpoint. The objective indication will be used in addition to the standard, but subjective method of visual indication by the user. Additionally, the sample's temperature is going to be monitored constantly because of the temperature's dependence on pressurization as well as its presumable effect on water potential. Lastly, we hope to increase the meaningfulness of our experiments by controlling the pressure in the chamber highly accurate and repeatable.

Methods

Quantitative measurement system

The quantitative measurement system we developed (figure 1) measures a leaf's efflux during a pressure chamber measurement by absorbing the sap and subsequently determining the caused displacement of a water column that already exists within the system. The sap enters the system through a membrane which acts as a semipermeable barrier in the manner that only water and no air can cross it. Absorption of the sap is caused by a pressure difference between the atmosphere and the inside of the system.
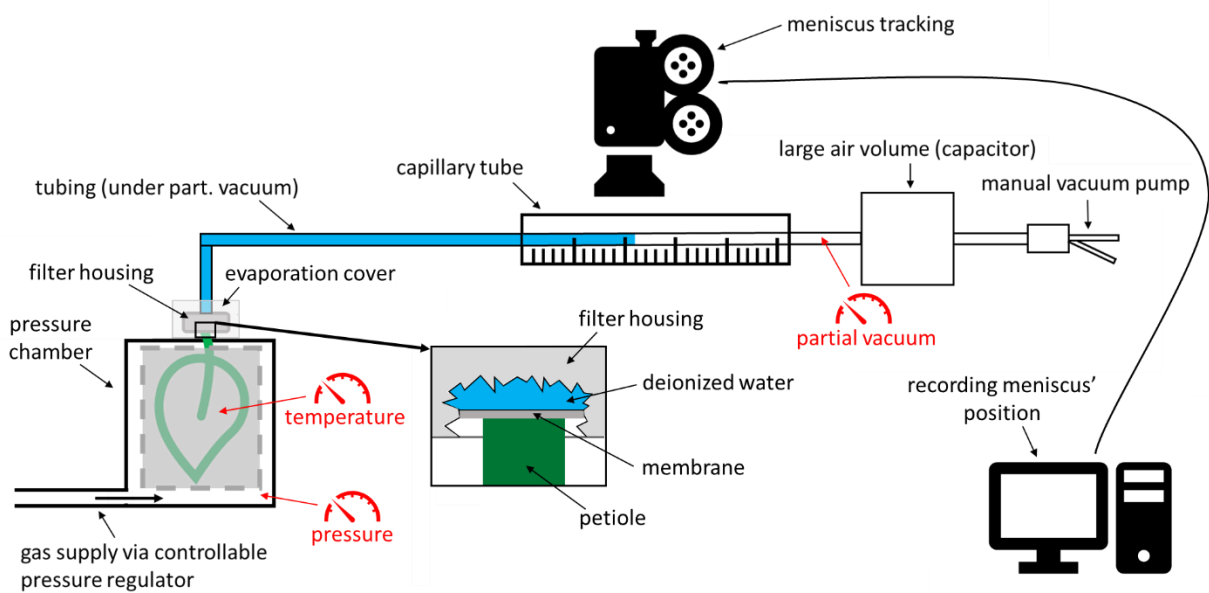
Figure 1: Overview of the quantitative measurement system.

A bagged leaf (aluminumized mylar bag (PMS Instrument Company, Albany, OR, USA)) inside a pressure chamber (core of a pump-up pressure camber (PMS Instrument Company, Albany, OR, USA)) is placed in contact with the quantitative measurement system via a membrane of a syringe filter which touches the end of the petiole (used filters were unidentified as to commercial source; for alternatives see appendix). We cut the filter housing open on one end to allow free access to the membrane. The filter

housing was surrounded by a plastic shield with pieces of wet paper towel along its inner edges used to reduce/prevent local evaporation (data not shown). The filter with the membrane is connected to tubing which ends at a manual vacuum pump (model 161.2174, Sears, Roebuck and Co., Hoffman Estates, IL, USA). The pump is used to reduce the pressure inside the system slightly underneath atmospheric pressure. The reductions range between 0.004MPa to 0.0065MPa. This means we introduced a so-called partial vacuum between 0.004MPa and 0.0065MPa within the system. In order to minimize changes in partial vacuum over time a glass vessel with a volume of 2 liter was inserted into the tubing to act as a capacitor. The pressure difference between the atmosphere and the inside of the system is the cause for water flow into the system. However, not only water also air flows along a pressure gradient. To ensure that no gas enters the system the membrane as the interface between atmosphere and system is key: from the inside of the system the membrane is in contact with water (deionized), and this water is strongly attracted to it since the membrane's perforation forms a multitude of capillaries. As long as the capillary forces are strong enough to withstand the pressure difference between the atmosphere and the inside of the chamber, the only entrance for air into the system is blocked by water. The ingress of water from the outside into the system however is not inhibited since it is equally attracted to the membrane as the water inside the system and will be absorbed as soon as it makes contact with the water from the inside due to the pressure gradient. This ingress displaces the water inside the system. The displacement can be recognized because there is a continuous water column between the membrane and the inside of a capillary tube. As capillary tube we used the barrel of a 50µl glass syringe (Hamilton Company, Reno, NV, USA). The small inner diameter of the syringe barrel causes little changes in water volume to provoke a considerable alteration in the position of the water meniscus within the barrel. The meniscus' end within the barrel is filmed (camera: CV-M10 SX, JAI Corporation, Copenhagen, Denmark) and software (Wong et al. 2009) indicates the end of the meniscus in the video footage as well as digitalizes its position. The digital position over time is recorded on a computer. In order to convert the change in digital position to

water volume, the system was calibrated by measuring the digital position of the 1μl marks on the barrel

of the syringe (which can be also seen in the video footage) with the software and dividing the position

change by the accompanied alteration in volume.

The pressure chamber is fed with nitrogen gas (N_2). A pressure regulator (QBS I, Proportion-Air,

Inc., McCordsville, IN, USA), controlled by a CR1000 datalogger (Campbell Scientific, Inc., Logan, UT, USA),

was used to conduct the desired pressurization of the chamber. The datalogger was also applied to record

the pressure inside the chamber (measured by the pressure regulator) as well as to initiate and record the

measurement of partial vacuum in the system and temperature of the leaf (measurement of partial

vacuum via a PX26-015GV pressure transducer (Omega, Inc., Norwalk, CT, USA); measurement of

temperature was via a 40 gage thermocouple (Omega, Inc., Norwalk, CT, USA) taped to the surface of the

lamina).

As part of system development, apparent water volume changes only associated with step

changes in partial vacuum were tested with the filter housing enclosed in a plastic bag with pieces of wet

paper towel inside to reduce/prevent evaporation. Step changes in true water volume were similarly

tested at constant partial vacuum by applying measured amounts of water to the membrane with the

needle of a 5μl syringe inserted through the plastic bag and applying droplet sizes of about 1μl.

Sample preparation

Fully expanded and mature grapevine leaves (variety Chardonnay) from greenhouse grown plants were

used for the experiments. After rehydration overnight, average leaf (lamina) weights were around 4.5g.

Rehydration with deionized water or dehydration by exposure to air has been done in various ways (e.g.,

leaves either attached or already detached from shoot). After re- or dehydration, leaves were treated with

two different protocols to ensure water potential equilibration: In the first protocol, they were enclosed

in aluminumized mylar bags (PMS Instrument Company, Albany, OR, USA) and put into another, larger

plastic bag containing wet paper towels and stored in a styrofoam box for at least 10 minutes, but usually a few hours before being used for measurement. Leaves were either attached or detached from the shoot. In the case of attached leaves, all other leaves remaining attached to the same stem were also enclosed in mylar bags. In the second protocol, leaves were detached from the stem and enclosed in mylar bags for at least 10 minutes before measurements started. The majority of leaves were treated with the first protocol. The bagged leaves had up to a few centimeters of petiole protruding.

Objective endpoint indication

When the meniscus' position within the syringe barrel was observed to begin moving (in the video footage) during the pressure increase in the chamber, the pressure was either completely released or held constant at the current value (see below sections for when which protocol was used). In the subsequent analysis of the data, a segmented linear regression was fit to the pattern of indicated sap volume over time. The outcome were two lines that represent two time-based segments. We were assuming little or no change in indicated volume in the first segment until the meniscus within the syringe has started moving, and after that a linear increase in indicated volume at least for a short period of time in the second segment. When volume data started to deviate from this linearity after a certain amount of time, it was not included in the regression anymore. The time at the intersection of the two regression lines was calculated and the accompanied pressure in the chamber used as the endpoint pressure. This objectively determined endpoint will be called the quantitative endpoint in the following. Statistical analyses were done using SAS statistical software (SAS 9.4, SAS Institute Inc., Cary, NC, USA).

Visual endpoint indication and protocols to compare visual and quantitative endpoints

For visual endpoint detection, the petiole was observed at a magnification between 25x to 40x on a dissecting microscope (Wild Makroskop M420, Wild Heerbrugg, Heerbrugg, Switzerland) equipped with a

video camera (E31SPM, Hangzhou ToupTek Photonics Co., Ltd., Hangzhou, China). The pressurization of the chamber as well as the measurement of the sample's temperature were conducted as described for the quantitative measurement system. The live recording from the camera as well as live monitoring of the chamber's pressure data – using the Loggernet software (Campbell Scientific, Inc., Logan, UT, USA) – were recorded as a single screen using Kaltura Capture (Kaltura, Inc., New York, NY, USA). By replaying the video, the first, clear sign of any fluid efflux from the petiole which was directly followed by additional efflux, was taken as the endpoint time and pressure (figure 2).[1] Since it was not possible to compare visual and quantitative endpoints for the same leaf at the same time, a set of 8 leaves representing a range of water potentials (-0.2MPa to -2MPa) were each measured 3 times consecutively, with 2 quantitative and 1 visual protocol in all combinations of order (manually stratified) (table 1).

Figure 2: Visual endpoint indication: The left picture shows the petiole without efflux about 2s before the endpoint was determined, the middle picture displays the moment the endpoint got indicated, the right picture shows the increase in efflux directly after the endpoint's indication (< 1s).

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[1] Example video (1st leaf of comparison between visually and quantitatively indicated endpoints): https://video.ucdavis.edu/media/Visual+Endpoint+Indication/1_r42jgv9r.

leaf	1. run	2. run	3. run
1	vis	quant	quant
2	quant	quant	vis
3	quant	vis	quant
4	vis	quant	quant
5	quant	vis	quant
6	quant	quant	vis
7	vis	quant	quant
8	quant	quant	vis

Table 1: Distribution of quantitative and visual measurements per leaf for the comparison between quantitative and visual endpoint indication.

Since some amount of water is lost from the leaf following efflux (either from absorption by the membrane in the case of a quantitative endpoint or potentially evaporation in the case of a visual endpoint), the visual endpoint was compared to a linear extrapolation or interpolation based on order, from the two quantitative endpoints. Hence, the visual endpoint was compared to the value predicted from the two quantitative endpoints corresponding to the same order of position as that of the visual endpoint for each leaf. For this experiment a pressurization rate of 0.003MPa/s was used. Data was collected at 2Hz and averaged to a 1Hz value. When the meniscus' position within the syringe was observed to begin moving during pressure increase, the pressure was held constant until efflux appeared to have stopped, and then released completely. We did similarly in the visual measurements of the first four leaves, but for subsequent samples we released the pressure considerably before efflux appeared to have stopped.

Testing pressurization with continuously applied rates and split regimes

Four different continuous pressurization rates were applied to pressurize the chamber (0.003MPa/s, 0.01MPa/s, 0.05MPa/s and 0.1MPa/s). 0.003MPa/s as the slowest rate was used as a control. Each leaf we tested was pressurized with all four rates (table 2). All rates instead of the control were used for quantitative as well as visual endpoint indication. The control was used only with quantitative endpoint

indication. A control measurement was taken between each pair of pressurizations with the higher rates, as well as in the beginning and at the end of each leaf's test protocol. This makes a total number of 10 pressurizations per leaf. The order of the applied rates was not randomized, so the protocol presented in table 2 was the same for each of the samples we tested. Similarly as for the visual endpoints, the control rate endpoints were used to establish a regression equation based on the order of measurements, and each test rate was compared to the regression's control rate value corresponding to the order of the test rate's measurement. We examined 10 leaves with water potentials between -0.15MPa and -2.1MPa. Data was collected in 200ms intervals without averaging and the pressure was completely released as soon as the meniscus' position within the syringe barrel was observed to begin moving during a pressure increase.

run	pressurization rate (MPa/s)	indication method
1	0.003 (control)	quantitative
2	0.01	quantitative
3	0.01	visual
4	0.003 (control)	quantitative
5	0.05	quantitative
6	0.05	visual
7	0.003 (control)	quantitative
8	0.1	quantitative
9	0.1	visual
10	0.003 (control)	quantitative

Table 2: Order of applied pressurization rates and the used method for endpoint indication in the investigation of continuously applied pressurization rates. The whole protocol was applied on each leaf that was tested.

We tested three different split pressurization regimes: Either 0.1MPa/s, 0.2MPa/s or 0.4MPa/s was applied in the first phase of the measurement until in all regimes 0.003MPa/s was used in the second phase until the endpoint had been reached. All regimes were applied on each leaf we tested. The first and last measurement of each leaf was a control, where the chamber got continuously pressurized with 0.003MPa/s (table 3). The order of the applied regimes was not randomized, so the protocol presented in table 3 was the same for each of the samples we tested. The two phases of pressurization were switched

approximately 0.1MPa prior to the endpoint determined by the first control run of each sample. Similarly as for the continuous application of one rate, the two control rate endpoints were used to establish a regression equation based on the order of measurements, and each test regime was compared to the regression's control rate value corresponding to the order of the test regime's measurement. In total, 11 leaves in a range of water potential between -0.15MPa and -1.9MPa were examined. Data was collected in 200ms intervals and not averaged afterwards. The pressure was completely released as soon the meniscus' position within the syringe was observed to begin moving during pressure increase.

run	pressurization rate (MPa/s)	indication method
1	0.003 (control)	quantitative
2	0.1 (1. part) | 0.003 (2. part)	quantitative
3	0.2 (1. part) | 0.003 (2. part)	quantitative
4	0.4 (1. part) | 0.003 (2. part)	quantitative
5	0.003 (control)	quantitative

Table 3: Order of applied pressurization regimes and the used method for endpoint indication in the investigation of split pressurization regimes. The whole protocol was applied on each leaf that was tested.

Efflux and the pressure difference between maximum pressure and the endpoint

To observe the efflux patterns after the endpoint had been surpassed, we analyzed the efflux data of the quantitative measurements from the comparison between visual and quantitative endpoint indication in more detail. In these measurements we initiated a constant pressure after the meniscus in the syringe barrel has been observed to start moving. For the time of constant pressure, we fitted double exponential functions to the volume data over time to get a mathematical approximation of the patterns (e.g., figure 3). The parameters of the double exponential functions were then used to compute the amount of efflux during the time of constant pressure. To get the total amount of efflux for every run, we added the small amount of sap outflow that occurred between the endpoint and the initiation of the constant pressure. The difference between the constantly held pressure and the endpoint pressure constitutes the pressure

difference between maximum pressure and endpoint. How fast the sap flows out at the cut end when the endpoint had been surpassed was determined by getting the slope of the line that approximated the second phase (efflux occurrence) in the segmented linear regression from the quantitative endpoint determination.
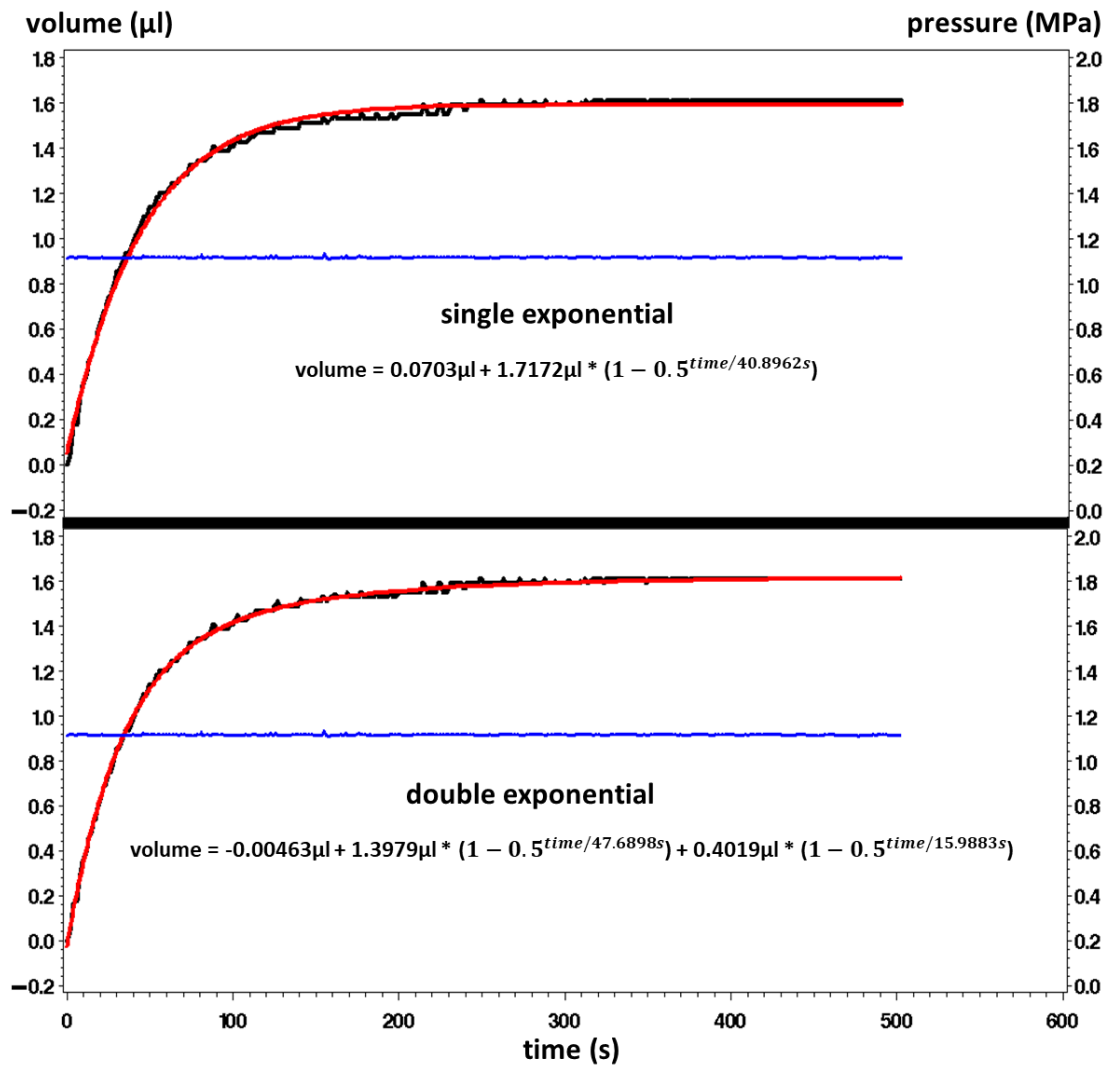
Figure 3: Example of a single and double exponential function (red) fitted to the volume data (black) for the time of constant pressure in the camber (blue) post endpoint. The data belongs to the 1st quantitative measurement of the 5th leaf of the comparison between quantitative and visual endpoint indication.

Results

Properties of the quantitative measurement system

The indicated volume by the quantitative measurement system was influenced by the amount of partial vacuum. An increase in partial vacuum led to an increase in indicated volume. Figure 4 illustrates this dependence by showing the rapid (data points 200ms apart) alteration in indicated volume when step changes in partial vacuum were applied. The range for partial vacuum was about 0.004MPa to 0.007MPa. This interval covers the range the system was operated in during actual measurements (0.004MPa to 0.006MPa). The applied changes in partial vacuum were accompanied with changes in indicated volume of a few tenths of a microliter. Even though volume changes of that height may be important to indicate the endpoint, it must be noted that the changes in partial vacuum over time during regular usage of the system were only a fraction of the applied changes in figure 4. Therefore, the changes in partial vacuum over time have been neglected during normal usage of the system.
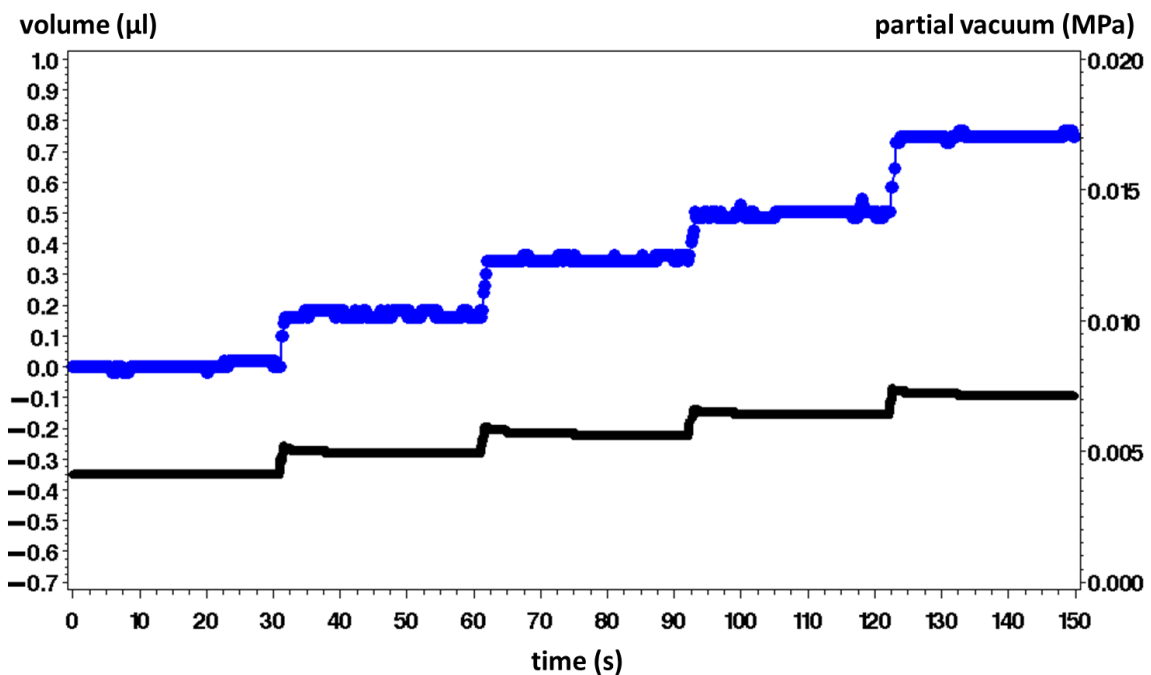
Figure 4: Quantitative measurement system - changes in indicated volume (blue) with changes in partial vacuum (black).

The system matched applied water volume closely. An example is shown in figure 5 where the indicated volume over the course of partial vacuum is demonstrated. In this experiment droplets of about 1μl were applied on the membrane. The step sizes were close to the targeted droplet size of 1μl, and the system showed a deviation of around 4% of the overall targeted volume of 5μl. Moreover, the response to the droplet application was rapid: Data was collected at 200ms intervals, and it took less than a second to detect the step changes. These performances are representative of a number of similar experiments that were conducted.
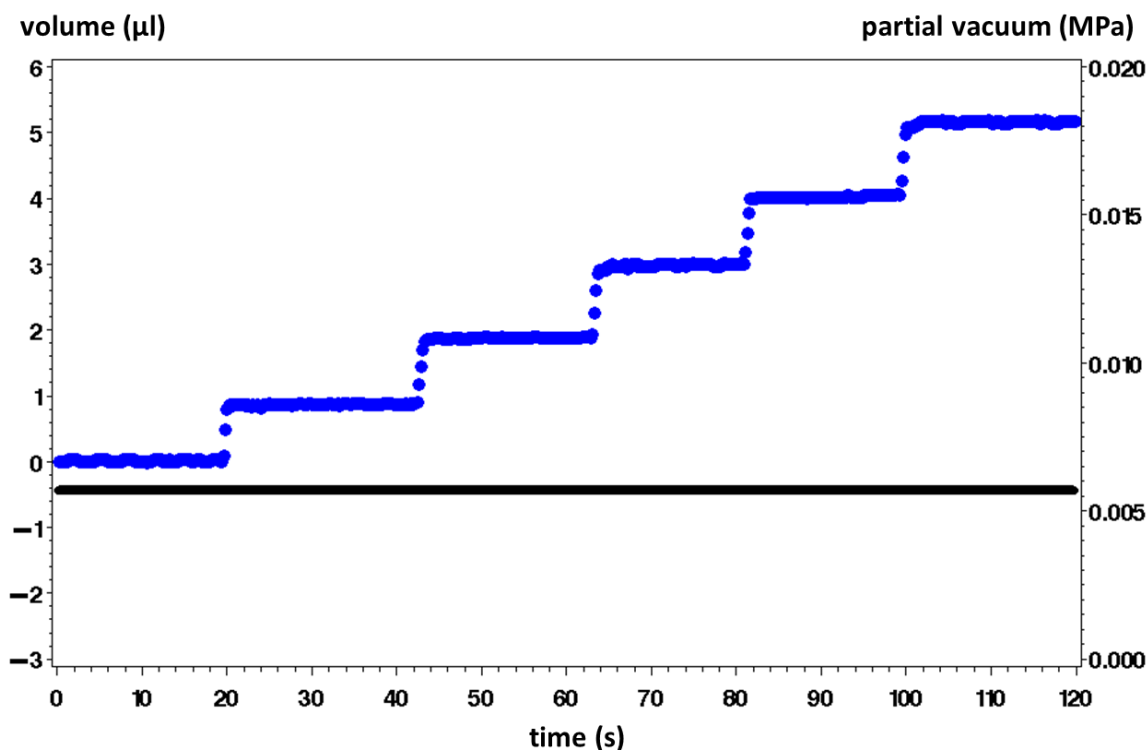
Figure 5: Quantitative measurement systems – response of indicated volume (blue) to droplet applications of about 1μl in size, over the course of partial vacuum (black).

Overview of a typical pressure chamber measurement

Figure 6 illustrates a typical pressure chamber measurement: After some time of only atmospheric pressure in the chamber, pressurization was initiated. During constant atmospheric pressure and during

24

the vast majority of the time of pressure increase, there was little (compared to the sap outflow that occurs later) to no change in volume detected at the petiole's cut end. This changed suddenly and a growing amount of efflux with time was measured. The growth appeared to be almost linear initially (for up to 20 seconds). So, simplifying we could note, that there were two phases of efflux in a pressure chamber measurement: "no efflux" and "positive efflux". Both phases were in general well fit by the segmented linear regression for the quantitative endpoint indication (figure 6, inset), with the pressure at the time of the line's intersection being defined as the endpoint pressure. The increase in pressure between the endpoint and when the pressure was held constant constitutes the pressure difference between maximum pressure and the endpoint. During the time of constant pressure following the endpoint, the total amount of indicated volume approached a plateau. The process could be closely fit with a double exponential function (not shown in figure 6). Whether the plateau has already been reached or not, the final pressure release back to zero, led to a constant value of indicated volume. Additionally, we want to make a note about the small gaps of volume data as seen in the inset picture in figure 6. The small gaps result from short delays in the meniscus tracking of the software. If such delays occurred, they lasted usually not longer than 1 second. However, if the delay caused the (artificial) conglomeration of volume data, only the first data point of the conglomeration has been kept and the others were discarded. Thereby, we wanted to assure that there wouldn't be false bias towards the conglomeration's particular volume level in the segmented linear regression, since it could diminish the accuracy of the subsequent quantitative endpoint indication.
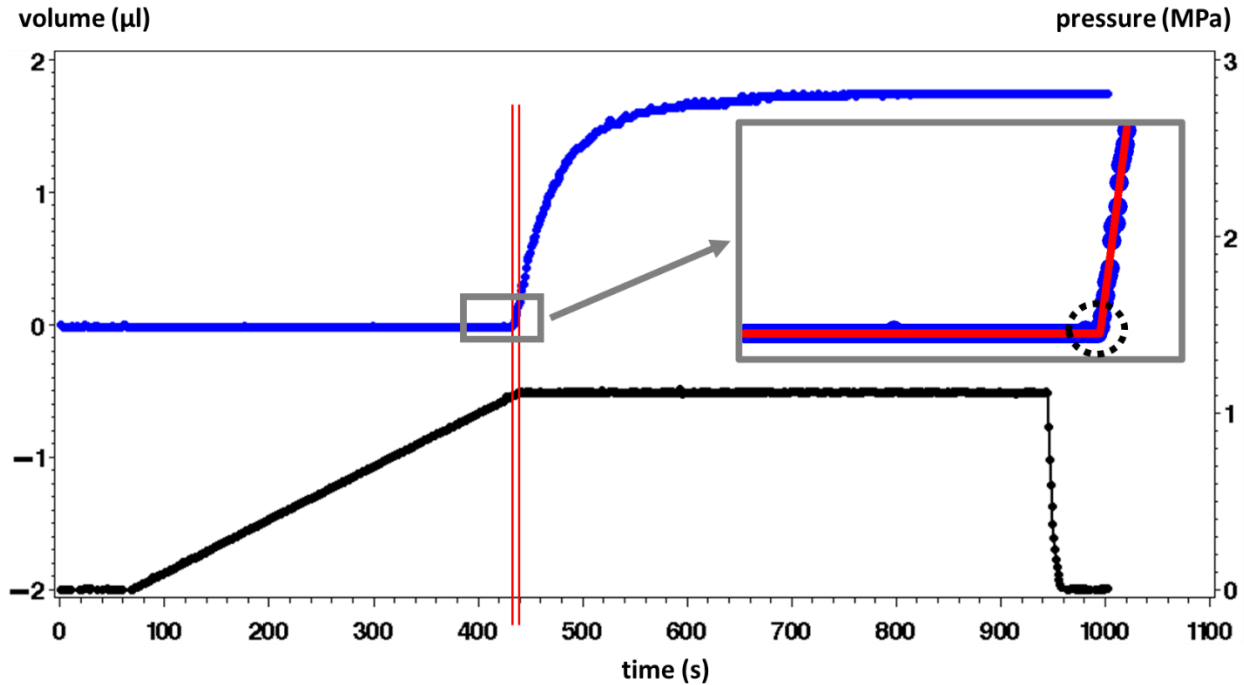
Figure 6: Quantitative measurement system - indicated volume (blue) and the chamber's pressure (black) during a typical pressure chamber measuremnt. The insert picture shows the two lines fitted as segmented linear regressions to the volume data (up to about 20s after the initial increase in volume). The two vertical red lines illustrate the pressure difference between maximum pressure and the endpoint when crossing the black pressure line. The data belongs to the 1st quantitative measurement of the 5th leaf of the comparison between quantitative and visual endpoint indication.

The temperature of the enclosed leaf (not shown in figure 6) generally increased during pressurization (exceptions were split pressurization regimes, see below), with the overall temperature increase depending on the rate and duration of the increase in pressure. Rapid rates of pressure increase exhibited a linear increase in temperature over time, whereas slow rates of pressure increase exhibited a nonlinear temperature increase, typically converging on a maximum temperature value, particularly when long time periods were required to reach the endpoint pressure for dry leaves (figure 7). Note that the maximum temperature increases observed – corresponding to high pressure increase rates and dry leaves – barely exceed 6°C (figure 7). The initial temperature increase (first 20s) was linear over time for all applied pressurization rates. Additionally, we found a strong proportionality between the applied pressurization rate and the average initial rate of temperature increase (figure 8), although with higher

variability as pressurization rate increased. The intercept of the regression line in figure 8 is not significantly different from 0.
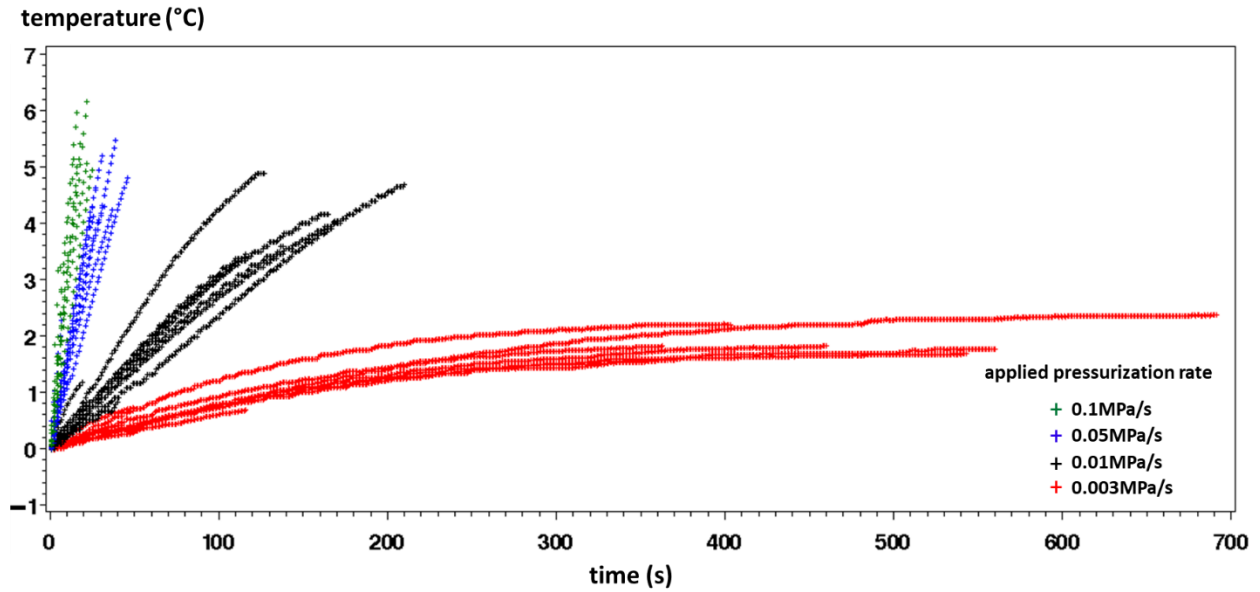
Figure 7: Temperature development from start to end (endpoint occurance) of pressurization for the quantitative measurements of each leaf from the test of different continuous pressurization rates. Since the control rate was applied 4 times on each leaf, we took the average of the four runs. Variation in the overall time of pressure increase for the same rate of pressure increase was due to the range of endpoint pressures of the samples (-0.15MPa to -2.1MPa).

rate of temperature increase (°C/s)

rate of temperature increase = 0.002568°C/s + 3.27991°C/s / (MPa/s) * pressurization rate

$R^2 = 0.8185$

pressurization rate (MPa/s)
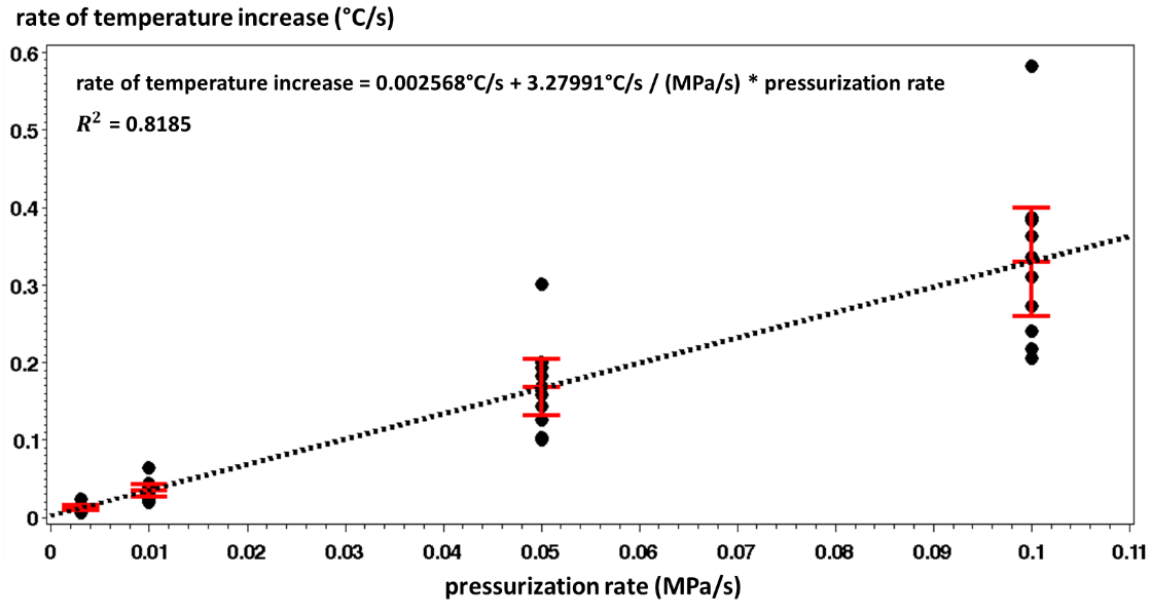
Figure 8: Initial rates of temperature increase (first 20s) on the sample's lamina for the quantitative measurements of each leaf from the test of different continuous pressurization rates. The red vertical lines are ± 2*standard deviation centered at the mean. The mean is indicated by the short horizontal line in the middle of the vertical lines.

Comparison between quantitative and visual endpoint indication

A comparison between quantitative and visual determination of the endpoint at a pressure increase rate of 0.003MPa/s revealed a very high correlation ($R^2 = 0.9993$), with an intercept and slope not significantly different from 0 and 1, respectively (figure 9).

quant. indicated water potential (MPa)

quant. ind. water potential = -0.022438MPa + 1.003598 * vis. ind. water potential

R^2 = 0.9993

vis. indicated water potential (MPa)
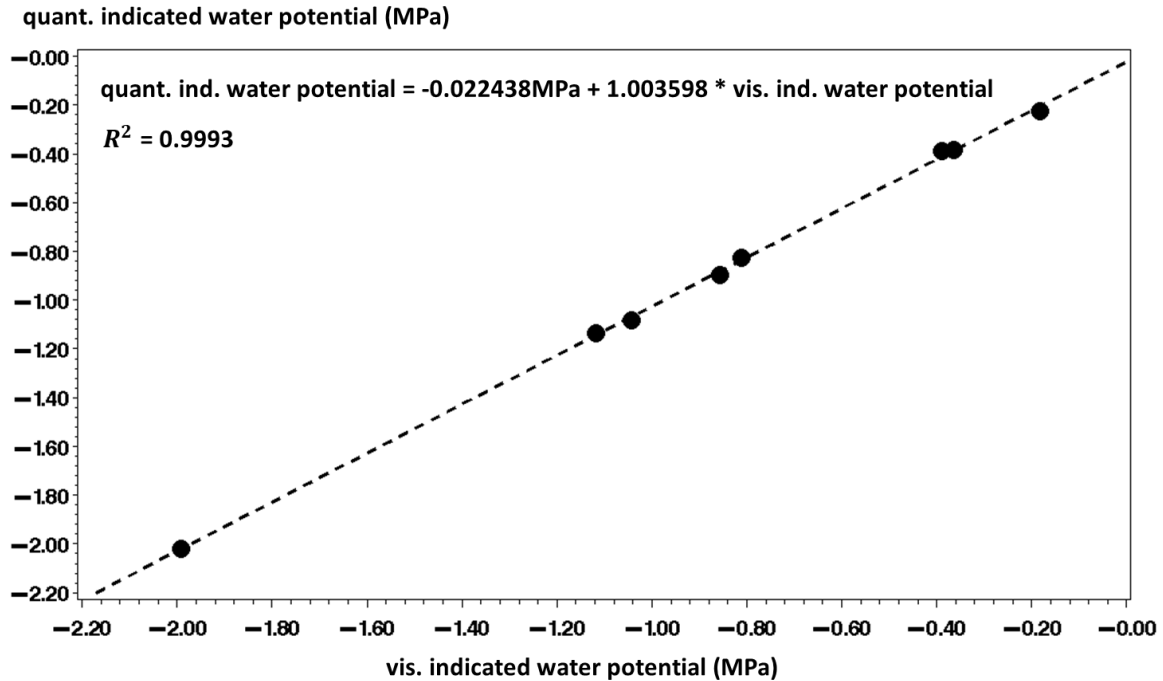
Figure 9: Relationship between quantitatively and visually indicated endpoints.

One continuous pressurization rate

Two ANCOVAs (table 4) reveal that the pressurization rate as well as the water potential of the control (0.003MPa/s) significantly influenced the indicated water potential when endpoints were determined quantitatively as well as visually. Additionally, we can conclude that neither the quantitative nor the visual indication show a significant interaction between the water potential of the control and the pressurization rate. However, the statistical significance of this result is only borderline for the quantitative indication. Lastly, it can be noted that if we exclude this non-significant interaction from the ANCOVAs the p-value of the pressurization rate's influence for the visual indication (formerly close to 5%), gets highly significant (as it is for the quantitative indication with and without the interaction).

ANCOVA | Cont. rates | Quant. Dependent variable: Water potential				ANCOVA | Cont. rates | Visual Dependent variable: Water potential			
source	DF	F-value	Pr > F	source	DF	F-value	Pr > F
pressurization rate	2	15.21	< 0.0001	pressurization rate	2	3.68	0.0403
water potential control	1	6873.59	< 0.0001	water potential control	1	5284.51	< 0.0001
water potential control * pressurization rate	2	2.91	0.0739	water potential control * pressurization rate	2	0.3	0.7424

Table 4: One rate continuously applied: ANCOVAs for the indicated water potential for both quantitative and visual endpoint determination.

There was a strong linear relationship ($R^2 > 0.98$) for both methods of indication between the water potentials indicated by the control measurements and the water potentials indicated by the test rates over the whole range of water potentials (figures 10 and 11).
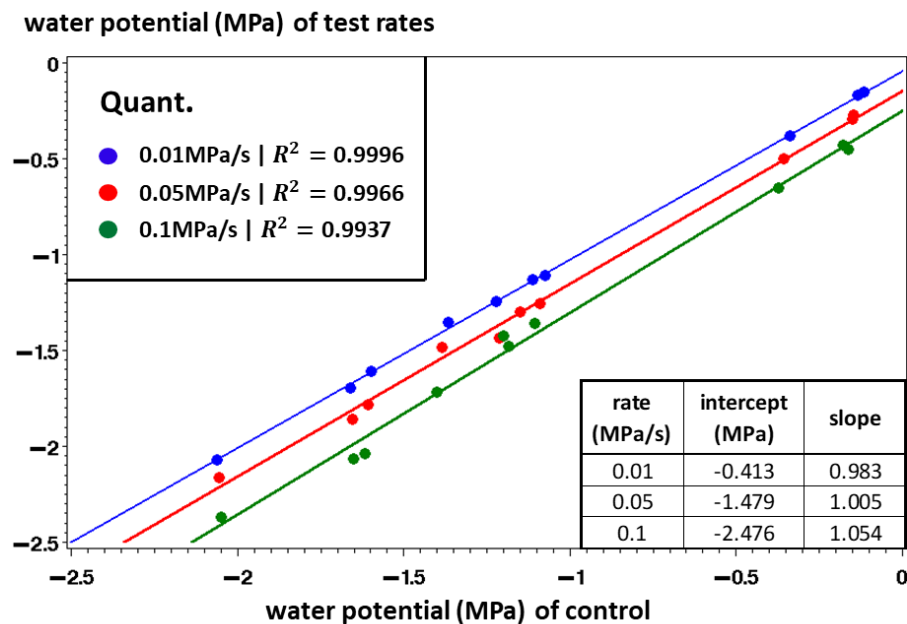
Figure 10: One rate continuously applied, quantitative indication: Relationship between water potentials of the three test rates and the control.

water potential (MPa) of test rates

rate (MPa/s)	intercept (MPa)	slope
0.01	-0.006	0.990
0.05	-0.656	0.985
0.1	-1.139	1.010

Visual
- 0.01MPa/s | $R^2 = 0.9995$
- 0.05MPa/s | $R^2 = 0.9985$
- 0.1MPa/s | $R^2 = 0.9885$

water potential (MPa) of control
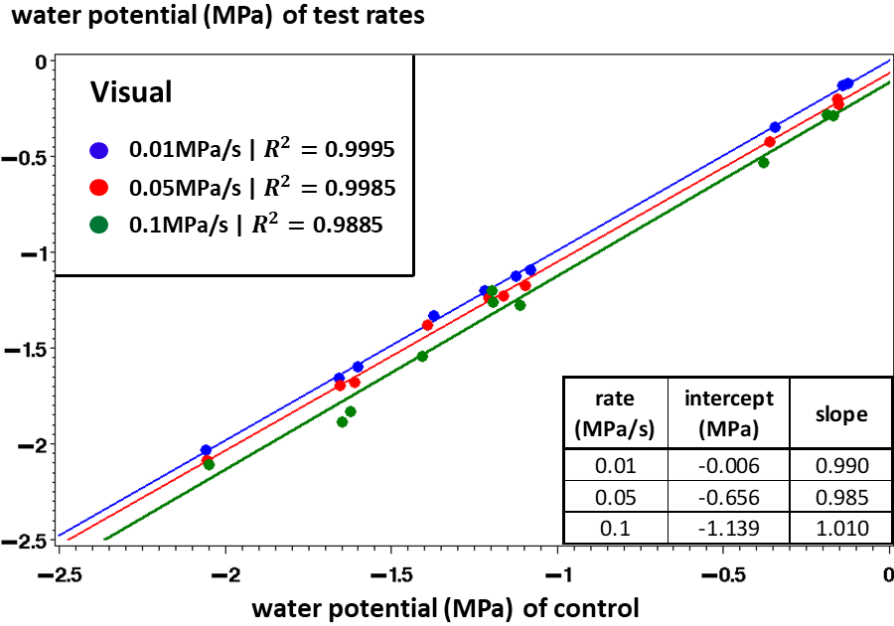
Figure 11: One rate continuously applied, visual indication: Relationship between water potentials of the three test rates and the control.

A more thorough analysis of the intercepts and slopes from the regression lines for the relationship between indicated water potentials by the test rates and the control (figure 10 and figure 11) has been conducted (table 5). As a result, the decrease in intercepts with higher pressurization rates for both the quantitative as well as well as the visual indication becomes apparent. We can note that when directly testing the intercepts against each other, we found that the intercepts of the two highest test rates did not significantly deviate from each other for the visual determination. However, both were significantly different from the intercept of the slowest test rate. For the quantitative endpoint indication, it was apparent that all intercepts of the test rates deviated significantly from each other. We found no significant differences between the slopes of the regression lines for the visual endpoint determination. However, for the quantitative method the slopes of the slowest and highest test rate deviated significantly from each other.

quantitative						
Rate (MPa/s)	Variable	Estimate	Standard Error	t Value	Pr > |t|	Test Grouping
0.01	Intercept	-0.413MPa	0.090MPa	-4.59	0.0018	a
0.05	Intercept	-1.479MPa	0.261MPa	-5.66	0.0005	b
0.1	Intercept	-2.476MPa	0.372MPa	-6.66	0.0002	c
Rate (MPa/s)	Variable	Estimate	Standard Error	F Value	Pr > F	Test Grouping
0.01	Slope	0.983	0.00723	135.99	0.0427	a
0.05	Slope	1.005	0.0209	48.09	0.8106	ab
0.1	Slope	1.054	0.02961	35.58	0.1083	b
visual						
Rate (MPa/s)	Variable	Estimate	Standard Error	t Value	Pr > |t|	Test Grouping
0.01	Intercept	-0.006MPa	0.102MPa	-0.06	0.9542	a
0.05	Intercept	-0.656MPa	0.166MPa	-3.95	0.0042	b
0.1	Intercept	-1.139MPa	0.484MPa	-2.35	0.0464	b
Rate (MPa/s)	Variable	Estimate	Standard Error	F Value	Pr > F	Test Grouping
0.01	Slope	0.990	0.00816	121.4	0.2669	a
0.05	Slope	0.985	0.01327	74.22	0.28	a
0.1	Slope	1.010	0.03846	26.25	0.8085	a

Table 5: One rate continuously applied – analysis of intercepts and slopes of the regression lines for the relationship between indicated water potentials by the test rates and the control (figure 10 and figure 11). The intercepts were tested against 0, the slopes were tested against 1. The grouping has been done by directly testing the intercepts, respectively slopes against each other for quantitative as well as visual endpoint indication.

We compared the water potentials indicated by the three test rates to the ones indicated with the control and found increasing deviations in indicated water potential between test rates and control with increasing pressurization rates (table 6). The higher the applied pressurization rate the more negative became the indicated water potential of a sample. Pressurization rate also influenced leaf temperature at the endpoint, with, on average, a higher leaf temperature at the endpoint with a higher pressurization rate (table 6).

leaf	average WP (MPa) of control rate	0.01MPa/s				0.05MPa/s				0.1MPa/s			
		deviation (MPa) of WP from control		deviation (°C) endp. T. test to control rate		deviation (MPa) of WP from control		deviation (°C) endp. T. test to control rate		deviation (MPa) of WP from control		deviation (°C) endp. T. test to control rate	
		quant.	visual	quant.	visual	quant.	visual	quant.	visual	quant.	visual	quant.	visual
1	-1.16	-0.019	-0.001	0.52	0.87	-0.150	-0.066	1.57	2.18	-0.294	-0.003	2.69	2.73
2	-1.09	-0.036	-0.011	0.29	0.64	-0.167	-0.076	1.18	1.73	-0.252	-0.164	2.22	2.42
3	-0.15	-0.040	0.003	0.12	-0.07	-0.127	-0.044	0.41	0.13	-0.255	-0.096	1.00	0.52
4	-0.15	-0.034	0.011	0.29	0.17	-0.146	-0.076	1.19	0.84	-0.288	-0.122	2.50	1.64
5	-1.21	-0.018	0.021	0.72	0.93	-0.222	-0.034	0.70	0.89	-0.224	-0.066	0.42	0.81
6	-0.36	-0.045	-0.005	-0.05	-0.20	-0.145	-0.064	0.26	0.10	-0.281	-0.154	0.54	0.37
7	-2.06	-0.007	0.029	0.54	0.88	-0.105	-0.033	0.53	1.18	-0.318	-0.059	0.93	1.33
8	-1.39	0.013	0.043	0.33	0.63	-0.098	0.010	1.02	1.44	-0.317	-0.134	2.00	2.31
9	-1.61	-0.009	0.006	0.57	0.83	-0.173	-0.067	0.60	1.06	-0.418	-0.210	1.30	2.05
10	-1.66	-0.033	0.001	0.77	1.07	-0.202	-0.040	2.37	2.59	-0.414	-0.235	2.78	3.40
		average (MPa)		average (°C)		average (MPa)		average (°C)		average (MPa)		average (°C)	
		-0.023	0.010	0.41	0.57	-0.153	-0.049	0.98	1.21	-0.306	-0.124	1.64	1.76

Table 6: One rate continuously applied: Differences in water potential (WP) as well as endpoint temperatures between the test rates and the control for quantitative and visual endpoint indication.

In order to evaluate if the deviations in temperature at the endpoint could be responsible for the accompanied deviations in water potential, we graphed the temperature deviations over the accompanied water potentials from the control for both methods of endpoint indication (figures 12 and 13). It became visible that the variation of the temperature deviations (standard deviation) was substantial: The standard deviations – computed for each test rate – were apparently of similar height as the differences in temperature deviation between the rates themselves (figures 12 and 13).

deviation (°C) in temperature at endpoint
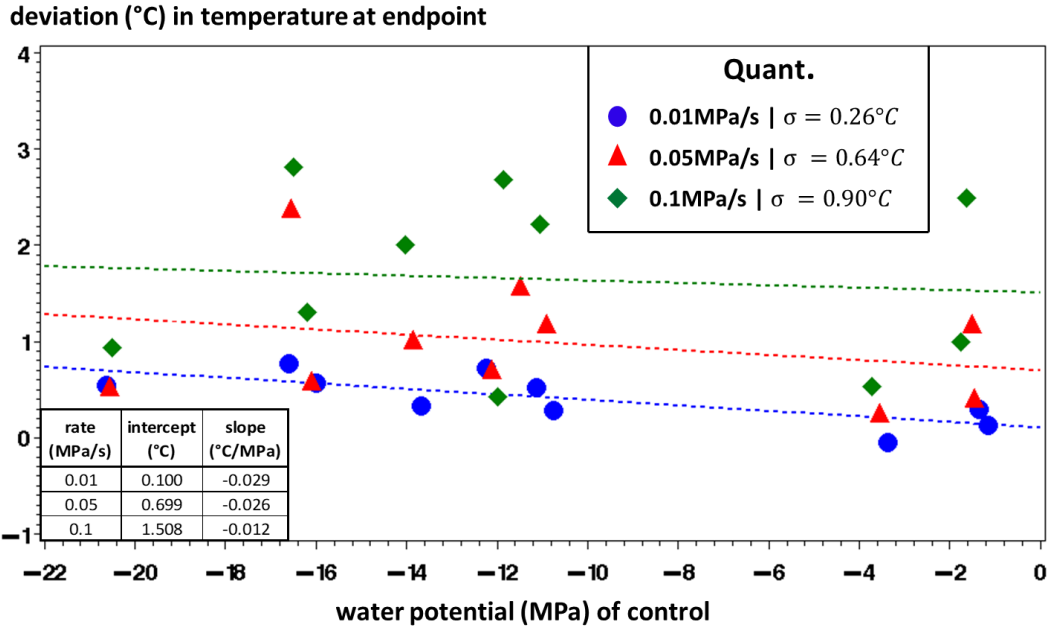
Figure 12: One rate continuously applied, quantitative indication: Deviation in endpoint temperature of the three test rates from the control over the corresponding water potentials of the control; intercepts and slopes from linear regression lines for each rate; standard deviations from linear regression.

deviation (°C) in temperature at endpoint
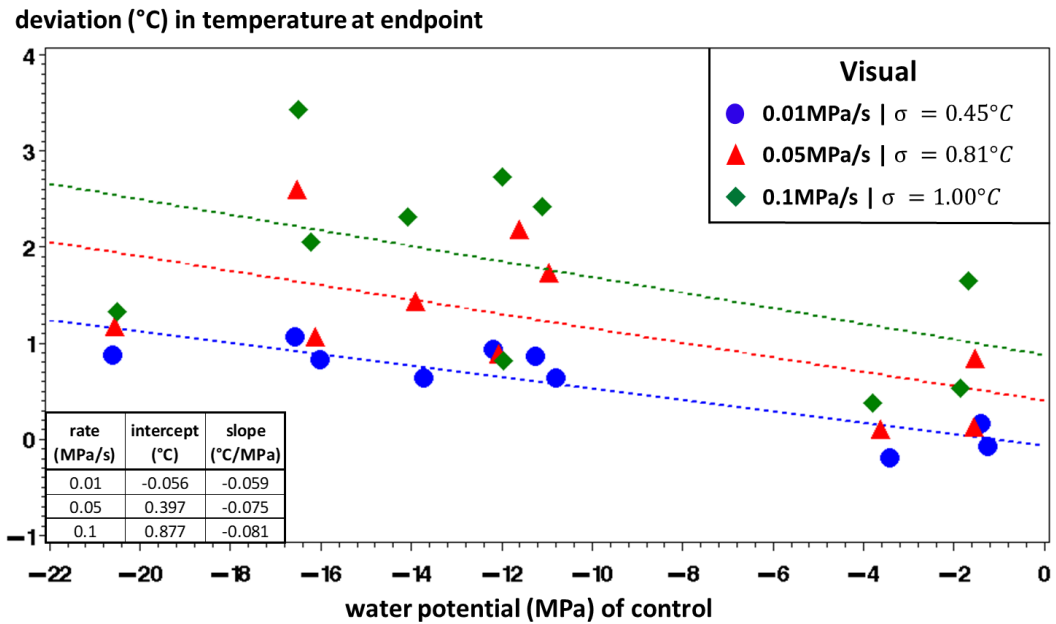
Figure 13: One rate continuously applied, visual indication: Deviation in endpoint temperature of the three test rates from the control over the corresponding water potentials of the control; intercepts and slopes from linear regression lines for each rate; standard deviations from linear regression.

Split pressurization regimes

Table 7 illustrates that the use of split regimes showed no significant influence of the pressurization regime on indicated water potential. Only the control's water potential was a significant influence since the interaction between the control's water potential and the pressurization regime wasn't significant either.

ANCOVA | Split regimes Dependent variable: Water potential			
source	DF	F-value	Pr > F
pressurization regime	2	0.05	0.9491
water potential control	1	40139.7	< 0.0001
water potential control * pressurization regime	2	0.17	0.8413

Table 7: Split pressurization regime: ANCOVA for the indicated water potential.

The close agreement between the readings of the three different test regimes is displayed in figure 14. Here, we can see a strong ($R^2 = 0.9993$) linear relationship between the indicated water potentials of all test regimes pooled together over the accompanied measurements from the control. Taking a closer look at the regression line, we can note that the intercept of about 0.017MPa is significantly different from 0 and that its slope of approximately 0.989 is significantly different from 1.

water potential (MPa) test regimes

water potential test regimes = 0.017054MPa + 0.989428 * water potential control

$R^2 = 0.9993$

- 0.1MPa/s + 0.003MPa/s
- 0.2MPa/s + 0.003MPa/s
- 0.4MPa/s + 0.003MPa/s
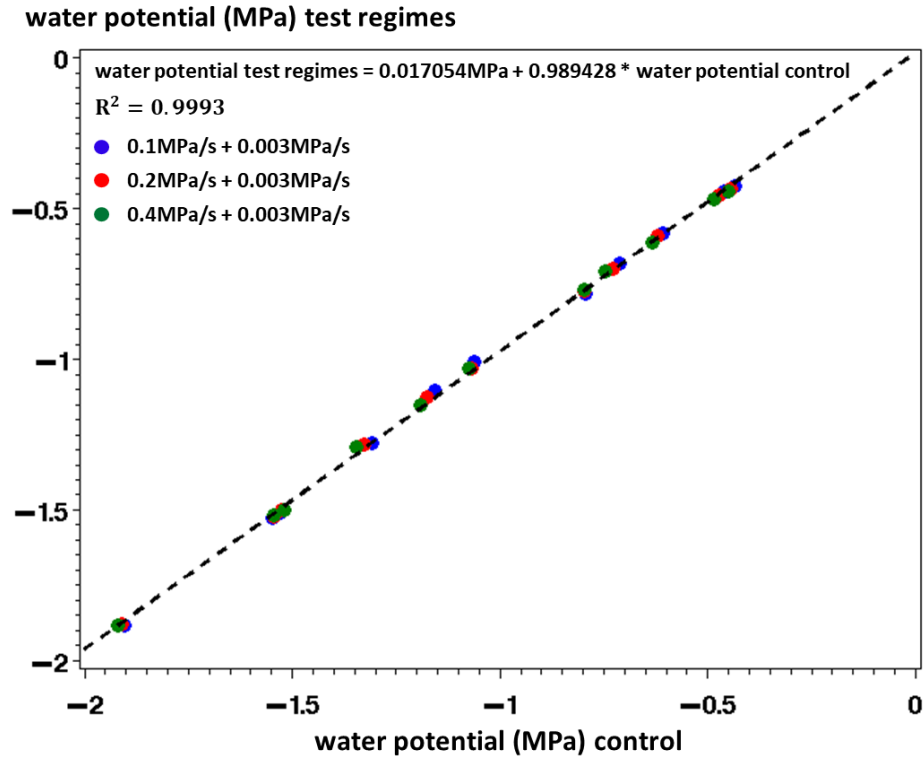
Figure 14: Split pressurization regime: Relationship in water potential between all pressurization regimes pooled together and the control.

As for the continuous application of only one rate, the pressurization regime influences the temperatures on the sample's lamina during the measurement (table 8). Though, the deviations at the endpoint between the three test regimes and the control are much smaller than for the continuous application of one rate. Additionally, we can note that all three rates we used in the first part of the pressurization were accompanied with fast increases in temperature. This changed instantly when the rates were switched: Pressurization with 0.003MPa/s in the second part of the measurement changed temperature far more slowly and both slight increases as well as slight decreases with respect to the temperature at the rates' shift have been observed.

leaf	ave. WP (MPa) of control rate	0.1MPa/s + 0.003MPa/s			0.2MPa/s + 0.003MPa/s			0.4MPa/s + 0.003MPa/s		
		delta WP. (MPa) test rate to control rate	delta T. (°C) test rate at split pressure to control rate at endp.	delta T (°C) at endp. test to control rate	delta WP. (MPa) test rate to control rate	delta T. (°C) test rate at split pressure to control rate at endp.	delta T (°C) at endp. test to control rate	delta WP. (MPa) test rate to control rate	delta T. (°C) test rate at split pressure to control rate at endp.	delta T (°C) at endp. test to control rate
1	-0.443	0.009	-0.065	-0.065	0.008	0.280	-0.140	0.006	0.615	-0.045
2	-1.912	0.023	0.192	0.442	0.032	-0.675	0.665	0.034	-0.613	1.028
3	-1.545	0.021	0.230	0.310	0.024	0.550	0.550	0.025	0.910	0.910
4	-1.524	0.021	0.098	-0.153	0.025	0.575	0.085	0.021	1.083	0.343
5	-1.327	0.030	-0.240	-0.410	0.044	-0.020	0.010	0.055	0.240	0.240
6	-0.796	0.012	0.015	-0.235	0.021	0.110	-0.300	0.027	0.285	-0.205
7	-0.472	0.018	-0.153	-0.282	0.015	-0.025	-0.275	0.015	-0.018	-0.178
8	-0.621	0.023	0.110	-0.390	0.029	0.100	-0.150	0.023	0.340	-0.240
9	-0.729	0.028	-0.045	-0.205	0.028	-0.080	-0.080	0.038	-0.045	0.035
10	-1.068	0.052	-0.228	-0.058	0.038	0.125	0.365	0.044	0.308	0.797
11	-1.175	0.054	-0.358	-0.157	0.049	-0.245	0.165	0.038	0.168	0.657
		average (MPa)	average (°C)		average (MPa)	average (°C)		average (MPa)	average (°C)	
		0.027	-0.040	-0.109	0.029	0.063	0.081	0.030	0.298	0.304

Table 8: Split pressurization regime: Deviations of water potential (WP) and temperature between test regimes and the control.

Consecutive measurements

We observed a significant decrease in water potential when measuring leaves consecutively (figure 15). The decline was found to be about 0.004MPa per subsequent measurement, but the linear relationship was rather weak with an $R^2 = 0.259$. We found the significant influence of the run number but can note that neither the individual leaf nor the interaction between leaf and run number had a significant effect on the decrease in water potential with consecutive measurements (data not shown). Similarly, the water potential of the sample showed no effect on its change in water potential with consecutive measurements (data not shown).

change in water potential (MPa)
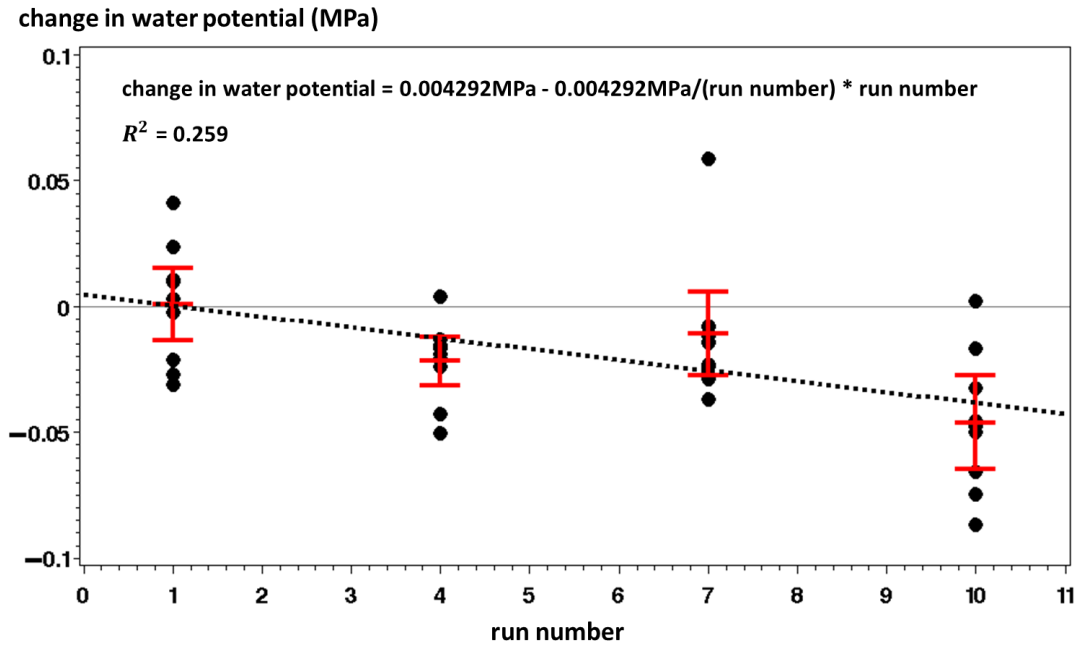
Figure 15: Development of the control's water potential from the test of the continuous application of one rate. The red vertical lines are ±2*standard deviation centered at the mean. The mean is indicated by the short horizontal line in the middle of the vertical lines.

Efflux and the pressure difference between maximum pressure and the endpoint

In table 9 we can see that for the comparison between visual and quantitative endpoint indication pressure differences between maximum pressure and the endpoint did not exceed 0.035MPa and got as low as 0.001MPa, as well as the fact that the different runs of the same leaf occasionally got exposed to very distinct values of this pressure difference. Smaller relative deviations than for the pressure differences between maximum pressure and the endpoint among the leaves, were found in the total amount of efflux, which ranged between 1.5µl and 16µl amongst the samples. The ratio of both – the efflux per pressure difference between maximum pressure and the endpoint – varied heavily among the samples (values between about 83µl/MPa and 5650µl/MPa). More homogeneous instead were the rates of initial efflux: 0.01µl/s was the lowest and 0.06µl/s the highest rate we measured. The observed initial efflux rates seem to cause visually distinct states at the cut end within a short amount of time. An example can be seen in figure 16. Here, two screenshots from the video footage of the first leaf from the

38

comparison between visual and quantitative endpoint indication show considerably different amounts of efflux within about 2 seconds. The efflux rate in this case was appr. 0.02μl/s (the average of the initial rates from the two quantitative runs of that leaf). It shall be mentioned that this is only a little more than half of what the average initial efflux rate across all samples from the comparison between visual and quantitative endpoint indication was (0.037μl/s).

leaf	number of quantitative measurement	average water potential (MPa)	pressure difference between max. pressure and endpoint (MPa)	total efflux (μl)	efflux per pressure difference between max. pressure and endpoint (μl/MPa)	rate of initial efflux (μl/s)
1	1	-0.98	0.018	1.491	82.8	0.017
	2		0.018	1.632	90.7	0.024
2	1	-0.97	0.021	1.947	92.7	0.048
	2		0.01	2.246	224.6	0.060
3	1	-0.38	0.013	3.034	233.4	0.039
4	1	-0.91	0.011	2.151	195.6	0.055
	2		0.008	2.401	300.2	0.046
5	1	-1.13	0.015	1.753	116.9	0.035
	2		0.015	1.913	127.5	0.029
6	1	-0.12	0.011	11.854	1077.7	0.052
	2		0.001	5.651	5650.5	0.048
7	1	-2.07	0.014	9.383	670.2	0.020
	2		0.035	16.084	459.5	0.010
8	1	-0.28	0.007	4.761	680.2	0.029

Table 9: Efflux analysis – data from the quantitative measurements of the comparison between visual and quantitative endpoint indication.
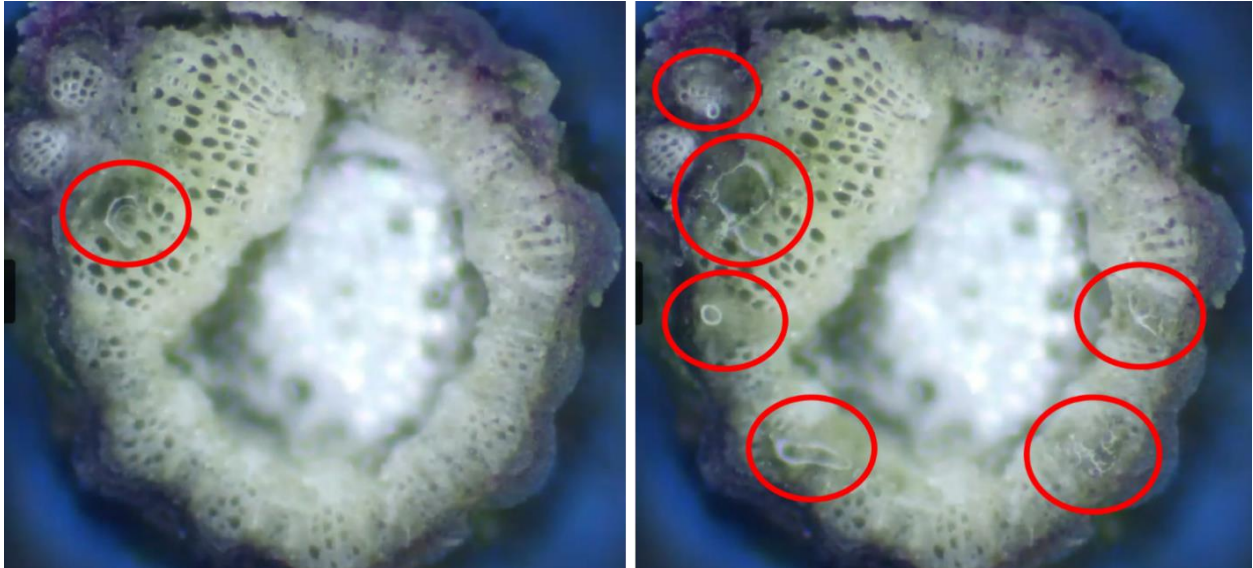
Figure 16: Two screenshots from video footage of the visual endpoint indication (1st leaf of the comparison between visual and quantitatively indicated endpoints). On the left is the cut end in the moment of endpoint indication, on the right is the same petiole about 2 seconds later. The efflux rate was approximately 0.02μl/s.

Discussion

The described quantitative measurement system determines applied water volume fast as wells as accurately and corresponds very closely to the visual endpoint indication at pressurization rates up to 0.01MPa/s. This is the first time that efflux over the whole course of a pressure chamber measurement has been quantified, which enables us to get an impression about what the numerous visual characterizations of the endpoint really constitute.

How can our observations from applying the quantitative measurement system be explained? Characteristic feature throughout our investigations was the presence of the two phases of efflux – a first phase when almost no change in indicated volume was detected and a second phase with positive efflux. In the first phase and before pressurization has been initiated, we can assume that the individual leaves have been at water potential equilibrium throughout their tissue. However, this should change as soon as we start to compress the gas in the chamber: When the pressure increases, we increase the water potential of the petiole's fraction outside the chamber (Dixon and Tyree 1984). Tissue outside the chamber is always necessary since the user must be able to visually observe the end of the petiole. The rehydration of the petiole's fraction outside the chamber requires water flow from the enclosed part of the leaf into this section. If we consider the assumption that leaves have different hydraulic compartments, we can conclude that the water flow towards the protruding part is not fed homogeneously throughout the tissue from the sample's section inside the chamber. Some compartments should lose more water than others which presumably breaks the initial water potential equilibrium. Though, it is very important to put the required water volume into context: The amount of tissue outside the chamber is only a fraction of the whole sample. In our experiments for instance, the protruding part was only about 7mm of petiole. So, the tissue inside the chamber should experience only a very small reduction in water content when rehydrating the protruding part. This in turn ought to be accompanied with only minor changes of water potential. Consequently, the compartments inside the

41

chamber would exhibit a nearly unchanged balancing pressure while they rehydrate the protruding tissue during pressure increase. So, the entirety of the enclosed part would experience balancing pressure at almost the same time and hence start extruding water almost simultaneously. The subsequent appearance of sap at the cut end of the petiole marks the beginning of the second phase of efflux we observed – the phase of positive efflux. We want to note here, that even though the changes in water potential due to rehydration of the protruding tissue are presumably small and negligible, the required water loss from the enclosed part lowers its water potential underneath the original state (where the whole sample was at an equal water potential). Therefore, the rehydration of the excluded tissue during pressurization represents an inevitable error in the pressure chamber technique.

By applying the quantitative measurement system together with the video footage for the endpoint indication, we observed the incidents around the endpoint in detail. As discussed in the introduction to this paper, the endpoint should ideally be indicated when the pressure exactly balances the tension in the xylem – which appears practically impossible. The indication by the user rather depends on the occurrence of efflux, which should be the result of a slight overpressurization beyond the pressure that exactly balances the tension in the apoplast. Therefore, this overpressurization to cause efflux is another inevitable error in the pressure chamber technique. However, we assume that the necessary overpressurization will be small: It appears that we can visually detect already tiny amounts of sap at the cut end of the petiole. We found visually distinct amounts of sap already after about 2 seconds at an efflux rate of about $0.02\mu l/s$. $0.02\mu l/s$ is only a little more than half of the average initial efflux rate we measured $(0.037\mu l/s)$. So, visually distinct states of efflux might occur already within 1s. We can estimate the scale of error if we consider the applied pressurization rate: An increase of $0.003MPa/s$ – like we used for the measurements in which we computed the initial efflux rates – would cause an error of $0.003MPa$ when it took the user 1s to differentiate states of no efflux and positive efflux at the petiole. Higher pressurization rates would increase the error. It seems possible however, that faster pressurization is accompanied with

higher efflux rates, which would counteract such increase in error with faster pressurization. Though, we must note that our estimation of the error due to overpressurization glosses over the aspect that at the instant of starting efflux, the sap menisci in the xylem might be located underneath the surface of the cut end and hence, some amount of efflux would be needed until the sap reaches the surface where we then can detect it. It would require a certain time span at a finite efflux rate (while the pressure is still increasing) until the sap reached the surface. Therefore, the error due to overpressurization would raise (during the required time span to bring the menisci to the surface) past our presented estimation. However, it seems to be practically impossible to locate where within the vessels the menisci are located when efflux first starts. Moreover, when taking the videos for visual endpoint indication, we saw that generally the first signs of efflux in a measurement come from only a fraction of all the vessels. Hence, only a fraction of the vessels needed to be filled until sap reaches the surface of the cut end. We therefore assume that the additional amount of efflux and thereby time to bring the sap to the cut's surface is negligible. We conclude that the technique's inevitable error due to necessary overpressurization to cause efflux should be small, especially when pressure is increased slowly around the endpoint.

Another observation we made through our investigations was the temperature increase in the chamber with increasing pressure. This is very much in alignment with the findings of (Puritch and Turner 1973). Though, the maximum temperature rises in our measurements, when one rate was continuously applied, were only about 6°C. This is considerably less than what Puritch and Turner (1973) found (up to 30°C) for similar rates. But the discrepancy is explainable, since the authors measured air temperature and we measured the temperature of a bagged leaf and directly on the sample's lamina: The leaf and the tape used to fix the thermocouple exhibit heat capacities which should dampen the effect of temperature changes of the gas surrounding the bagged sample. Furthermore, our leaves were enclosed in a mylar bag which should additionally insulate the sample. These considerations seem to align with investigations from Wenkert et al. (1978), who found temperature rises in the same magnitude like ours when they

measured actual leaf temperature on bagged samples. Additionally, we observed higher temperature increases during measurements with higher rates. This is expected taking the temperature rise due to the heat of compression into account and considering that a faster pressurization not only enhances the compression of gas, but that it also reduces the available time for counteracting processes like heat transfer thorough the chamber's walls. Another aspect we found about the temperature increases was the strong linearity between the average initial rate of temperature increase (first 20 seconds) and the rate of pressurization. Here, the heat of compression is central again. Its influence in isolation should lead to a linear relationship between the speed of temperature increase and the speed of pressure increase, since it would be the only effect on temperature. But in reality, counteracting processes like the heat transfer through the chamber's walls influence temperature as well and should lead to a deviation from such linearity. Counteracting processes should get more influential the faster the temperature inside the chamber rises (due to a faster pressurization rate) because of the resulting higher temperature differences between the inside of the chamber and the air outside the walls. But the time span of 20 seconds to determine the initial rates might not be enough for the counteracting effects to have considerable influence. Hence, the observed linearity between the initial rate of temperature increase and the pressurization rate in our investigations.

Even though not central to our study, we want to point at two additional observations we made: First, we found that when the endpoint did occur and we subsequently held the pressure constant rather than completely releasing it, the increase in indicated volume declined with time. This is in alignment with what has been reported previously in the literature (e.g. Tyree and Dainty (1973)). Secondly, and advancing the first observation, we found very close agreement when fitting double exponential functions to the indicated volume post endpoint. This is in line with Zwieniecki et al. (2007) – who found strong accordance between double exponential functions and rehydration patterns of leaves – when assuming similar underlying mechanisms for patterns in rehydration and dehydration.

44

Consecutive measurement of a sample led to a slight but significant decrease in water potential. This is reasonable since efflux at the endpoint should always be accompanied with water loss – at least due to evaporation. Though it seems important to not overestimate the magnitude of this result: The decrease of 0.004MPa/run is small, and we must take into account that in using the quantitative measurement system we absorbed all the efflux that occurred at the surface. Using the pressure chamber visually, should lead to less of a water loss and hence should reduce the value of 0.004MPa/run even further. Moreover, the relationship between the reduction in water potential and the number of measurements was weak ($R^2 \approx 0.25$) – it seems that the measurement variability is higher than the observed effect itself. Of course, there might be a species dependency of these values and therefore ongoing investigations are needed, but consecutive measurements of the same sample might not be very detrimental.

Integral part of our investigations was the test of different continuously applied pressurization rates. Fundamentally, we indicated decreasing water potentials with higher pressurization rates for the visual as well as for the quantitative endpoint indication. The same effect was observed in the literature's most comprehensive study about pressurization rates from Naor and Peres (2001) as well. The authors showed species dependent changes between -0.8MPa and -1.9MPa for an alteration in pressurization rate of 1MPa/s. In our investigation on grapevine the alteration for the visual indication was about -1.5MPa per 1MPa/s, and approximately -3.1MPa per 1MPa/s when quantitatively indicating the endpoint. So, the magnitude of the decrease we observed for the visual indication lies within the range the authors showed for deciduous trees. Even though the decrease that resulted from our quantitative indication is not of unreasonable height compared to what Naor and Peres (2001) showed, there seems to be a difference between the two methods of indication depending on pressurization rate. We will deal with the possible explanation for this difference later, because first we want consider temperature effects as they could have caused the general trend of decreasing water potentials with increasing rates independent from the

way of indication. We found that the decreases in water potential were accompanied with higher temperatures at the endpoint, and so the question arose if these temperature increases could be the reason for the alteration in water potential. Tyree et al. (1974) tested the influence of temperature on water potential. They found decreases in water potential with increases in temperature only when the measured shoots were dryer than about -1.8MPa. The temperature effect increased the dryer the samples became. They attributed this to the temperature dependency of the osmotic potential, which they assume should become more influential the lower the turgor pressure gets (Van't Hoff's principle). If the authors measured samples that were better hydrated instead (water potential > -1.8MPa) they reported the opposite response to temperature. Almost all samples we measured had a water potential > -1.8MPa, so our data showed the opposite relation between temperature and water potential to what the literature suggests. Of course, there could well be a species dependency of the temperature's influence and therefore the particular values of water potential that mark the change in the response's direction might not be universal. But it seems reasonable to assume that generally the influences of turgor and osmotic potential are similar among plants. Hence, the directions of temperature effect as described by Tyree et al. (1974) would be valid for our test species (grapevine) as well. So – assuming that the concepts proposed by Tyree et al. (1974) are right – our findings cannot be explained by the literature. And there is another aspect in our data that doesn't align with Tyree et al. (1974): Their reported magnitude of temperature dependence is far smaller than ours. The most extreme change in water potential with temperature they showed was only about a tenth of the highest change we observed when quantitatively indicating the endpoint and about a fourth when visually determining the reading. Additionally, we want to point at another aspect in our data showing that temperature cannot be the explanation for our observations: The temperature deviations at the endpoint between the test rates and the control had standard deviations that were similarly high as the differences between the rates themselves. So, if we assume that these temperature deviations – as the measure for the temperature increases with higher rates – were the

responsible factor, then their variation should cause similar variation in the deviations in water potential. However, we observed very distinct and strongly linear patterns in the water potential indicated with different rates over the whole range of measured water potentials. So in conclusion, it seems rather more likely that there is no causality between the temperature increases and the decreases in indicated water potential that we found. As discussed earlier, greater temperature rises should be the result of faster pressurization and the significant influence of the pressurization rate on indicated water potential when applying a continuous rate could have mechanisms other than temperature effects. But what other mechanisms could be the cause? One might be the increase in overpressurization with higher rates as discussed earlier, though it seems unlikely that it accounts for all of the magnitude in deviation we observed. The measured deviation from the control of more than 0.3MPa (absolute value) at a rate of 0.1MPa/s for the quantitative indication is too high, considering distinct amounts of efflux occurring presumably within 1 second. For visual endpoint indication the deviations between test rates and the control were lower. But the average deviation of about 0.12MPa (total value) at 0.1MPa/s pressure increase appears too high as well in order to be completely attributed to overpressurization. We used video recordings for the visual determination of the endpoint, which allowed us to go freely back and forth in time within fractions of a second as well as to persist at a certain time and compare the picture to previous states. We assume that such technical support of the visual observation should decrease the amount of time required to determine changes in sap appearance considerably below 1 second. And hence, we assume that overpressurization cannot completely explain the decreases in water potential with higher pressurization rate that we found in both methods of indication. Another possible explanation for our observations was discussed in the introduction of this paper. It is the fact that when visually indicating the endpoint, the user needs a certain amount of time for both processing the information of efflux occurrence and subsequently shifting the line of sight towards the pressure gauge to read the pressure. Higher rates would mean a higher pressure increase during the required time span. But this

mechanism should be ruled out in both methods we used to determine the endpoint: When quantitatively measuring the samples, the endpoint indication was done objectively without any involvement of the user during the measurement. And since the visual endpoint indication has been conducted via recordings where we could go freely back and forth in time, the described delay ought to be nonexistent in the visual indication as well. However, when considering the more pronounced decreases in indicated water potential from the quantitative indication in comparison to the visual indication, another possible explanation for the dependency of indicated water potentials on pressurization rate – at least for the quantitative method – comes into mind: There could be a delay in the detection of the initial sap outflow. At first, it might take the outflowing sap a certain amount of time (during which the pressure is still increasing) to enter the capillaries of the membrane, where it then can be absorbed by the system. After this initial ingress, subsequent efflux might be continuously connected to the inner part of the system and the delay in detection would disappear. Such issue could be the reason for the observed differences between visual and quantitative endpoint indication at higher pressurization rates. Though, we have no proof for this explanation: When applying the droplets on the membrane to test the system's performance, we had no information about the exact time when the water first touched the membrane. It would presumably require a very sensitive application and measurement system to detect the initial contact between droplet and membrane. However, another worthwhile approach to explain the decrease in indicated water potential with higher pressurization rates for both the quantitative as well as the visual indication might be to think about the influence of the tissue outside the chamber again: Rehydration of the outside tissue should happen at a finite speed. If the pressure increase inside the chamber was faster than the provoked rehydration of the excluded tissue, then hydration lagged more and more behind the faster we raise the pressure. This implies that at a certain pressure inside the chamber, the enclosed part of the leaf (which supplies the water for rehydration) had lost less water when the pressure was raised fast than when it was raised slower. Hence, the enclosed part was at a higher water potential. For the

processes within the leaf we could thereby infer, that when the pressure inside the chamber that balances the tension in the apoplast is exceeded and efflux starts, the enclosed part of the leaf would be at a higher water potential when pressure was raised fast than when it was raised slower. If the occurring efflux was not completely absorbed by the insufficiently rehydrated protruding tissue before it could appear at the surface of the cut end, then efflux would be detected at a higher water potential of the enclosed part when the pressure was raised fast than when it was raised slower. For this to happen, the xylem vessels needed to constitute a lower resistance to water flow than what the tissue's rehydration exhibited since then the sap flow would concentrate on the vessels and not on the rehydration paths. However, even if that was the case, we would indicate higher water potentials with faster pressurization. So, the rehydration of the excluded tissue cannot explain the decreases in indicated water potential with faster pressurization either. However, an aspect that struck us in general, was the strong linearity (proportionality) between pressurization rate and indicated water potential in most of the cases presented by Naor and Peres (2001) as well as in our investigations. This makes a time lag likely as an underlying mechanism: If the time lag was constant or linearly related to the pressurization rate, then the pressure increase during the lag would lead to a decrease in indicated water potential that is directly proportional to the pressurization rate. The time lag might occur between the start of efflux and its indication at the cut's surface, or between the pressure in the chamber and the provoked response of the plant tissue. Our reasoning concerning the possible issue of the quantitative measurement system as well as concerning overpressurization as an inevitable error in the pressure chamber technique fit into the category of a time lag as underlying mechanism. We were not able to judge the proposed scenario about the quantitative system's possible issue, but for the overpressurization we concluded that it couldn't explain all of the deviations between rates. So, either our understanding of overpressurization is wrong, respectively incomplete, or there might be another dynamic process that additionally delays the indication of the endpoint with higher pressurization rates.

We also tested split pressurization regimes where a high rate in the first part of the measurement was followed by a slow rate in proximity to the endpoint. Our data shows that the initially applied rate had no effect on the indicated water potential and that the measurements of all tested split regimes agreed closely to the control (0.003MPa/s continuously applied). This brought up the argument that it might be predominantly the endpoint's indication which is influenced by different pressurization rates: The three regimes differed largely in pressure increase during the first part of the measurement, but all had the same slow increase starting about 0.1MPa prior to the endpoint. So in fact, our investigations appear to indicate that the speed of pressurization almost only matters in close proximity to the endpoint. More striking we could state that our data suggests that for the accuracy of the pressure chamber measurement it doesn't really matter how we get close to the endpoint as long as we raise the pressure slowly when almost there. This implies that if a time lag was responsible for the influence of pressurization rates in general, then the lag probably concentrates on the processes between balancing pressure and the endpoint's indication and not on incidents earlier in the measurement. However, our findings contradict Naor and Peres (2001), whose data illustrated that a change in the initial pressurization rate was indeed influential and led to an increase in indicated water potential of almost 0.6MPa per 1MPa/s raise of the initial rate. The only reason we can think of to cause an increase in indicated water potential with higher initial rates is the lower rehydration of the excluded tissue because of the overall faster measurement, as discussed earlier. Though, this effect should have occurred in our investigations as well and therefore provoked a similar response. Of course a species dependency of the rehydration process and different amounts of tissue outside the chamber (it is not stated from Naor and Peres (2001) how far petioles protruded) cannot be ruled out, but it appears unlikely, that these factors alone could account for the differences to our study. So, with our current knowledge it appears as if we cannot explain the difference between Naor and Peres (2001) and our investigation in split regimes. Finally, we want to examine another observation we made when testing split regimes: On average, there were similarly high,

50

slight increases in indicated water potential in all split regimes compared to the continuously applied control. The fact that the increases were of similar height, is reasonable since we found that the pressurization regime had no influence on the measurement, but the fact that the deviations were positive is striking. Apparently, there seems to be some influence that all our split regimes possessed, though we don't know what a possible mechanism could be. However, it appears important to put the magnitude of these increases into context: The average heights were only about 0.03MPa in all three regimes – a value that is negligible for most practical applications. Further investigations and the test of different species is necessary, to increase our understanding and to draw a final conclusion about split pressurization regimes. Though our data suggests that split regimes might be more favorable than the continuous application of one rate since they can save time and still achieve high accuracy.

Conclusion

The described system allows the accurate quantification of efflux during pressure chamber measurements which enabled the objective indication of the endpoint. Objectively indicated endpoints showed close agreement to the visual indication up to a pressurization rate of 0.01MPa/s. We challenged existing uncertainties about different pressurization rates and regimes for usage in the pressure chamber. Our investigations showed a decrease in measured water potentials with higher continuously applied rates. The underlying mechanisms are not clear, but a time lag seems likely. Split pressurization regimes appear to be a time efficient way to take measurements at high accuracy. Therefore, they might be favorable with respect to the continuous application of one rate, but further examination and the test of more species is necessary to draw final conclusions.

Appendix

The filters used in the quantitative measurement system were unidentified as to commercial source, but commercially available syringe filters which also allow direct access to the inlet face of the membrane were found and tested. We compared three different types of hydrophilic membranes (Sterlitech Corporation, Auburn, WA, USA) in a 13mm Swinnex Polypropylene Filter Holder (Sterlitech Corporation, Auburn, WA, USA) against the unidentified type we used. The three membranes – Nylon, Polyethersulfone, and Nitrocellulose Mixed Esters (MCE) – had a pore size of 0.45µm and were rated at bubble pressures of 0.24MPa (Polyethersulfone and MCE), and 0.21MPa (Nylon) respectively. Three properties were evaluated: 1) the magnitude of change in indicated volume with change in partial vacuum, 2) long-term stability of indicated volume at a constant partial vacuum of about 0.006MPa, and 3) the response time with a volume change caused by applying a 1µl droplet of water. Data was collected in 200ms intervals and not averaged.

To test the magnitude of change in indicated volume with change in partial vacuum we applied sudden changes in partial vacuum within a range of 0.004MPa and 0.011MPa (e.g., figure 17). When the partial vacuum had stabilized after a change, the corresponding volume was determined and a regression between partial vacuum and volume conducted (e.g., figure 18). A rigid and predictable behavior of the membranes is desirable, so small changes in indicated volume and a relationship close to linearity would be favorable properties. As a measure we took the slope of the regression lines for indicated volume over partial vacuum and the coefficient of determination (R^2) of these fits for each type of membrane (table 10). All R^2s were high (>0.98), so linearity appears to be given in each type. When comparing the slopes, we can see that they are all in the same magnitude. Considering their standard errors, suggests that there might be considerable differences between them. But we have to take into account that the basis for our investigation here is only 1 repetition for each membrane type. So, drawing a final conclusion about

possible differences between the membranes in terms of changes in indicated volume with changes in partial vacuum requires more data, but similar performance levels are suggested by our examination.
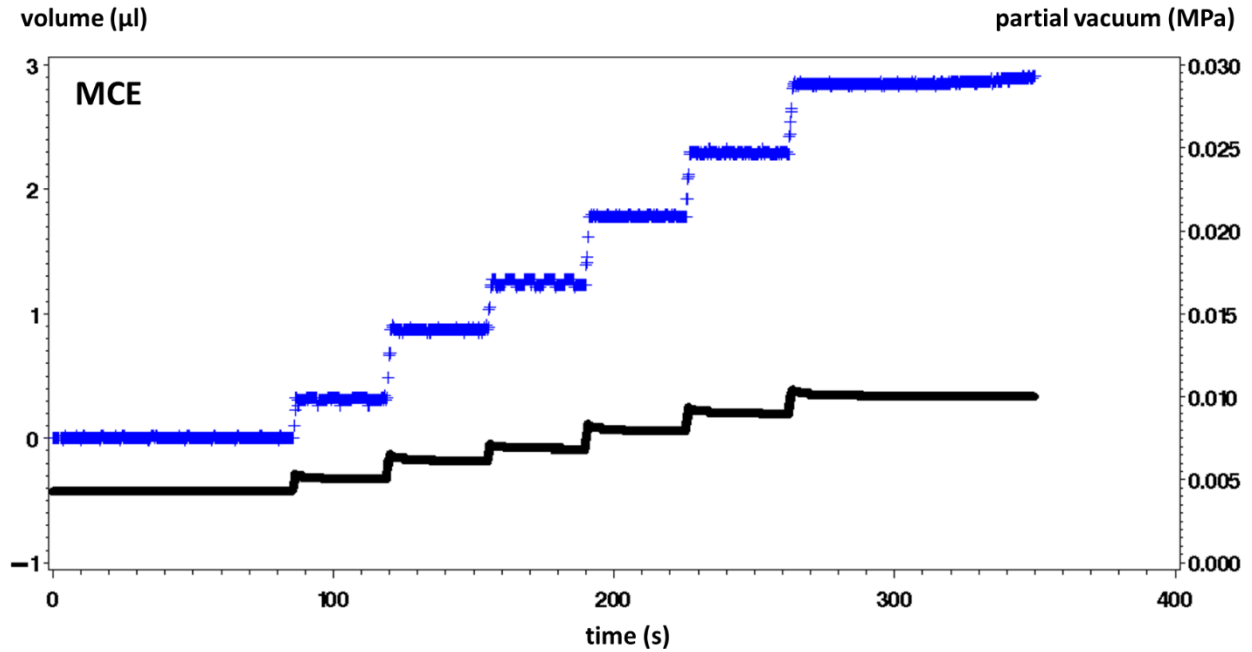

Figure 17: MCE membrane, changes in indicated volume (blue) with changes in partial vacuum (black).
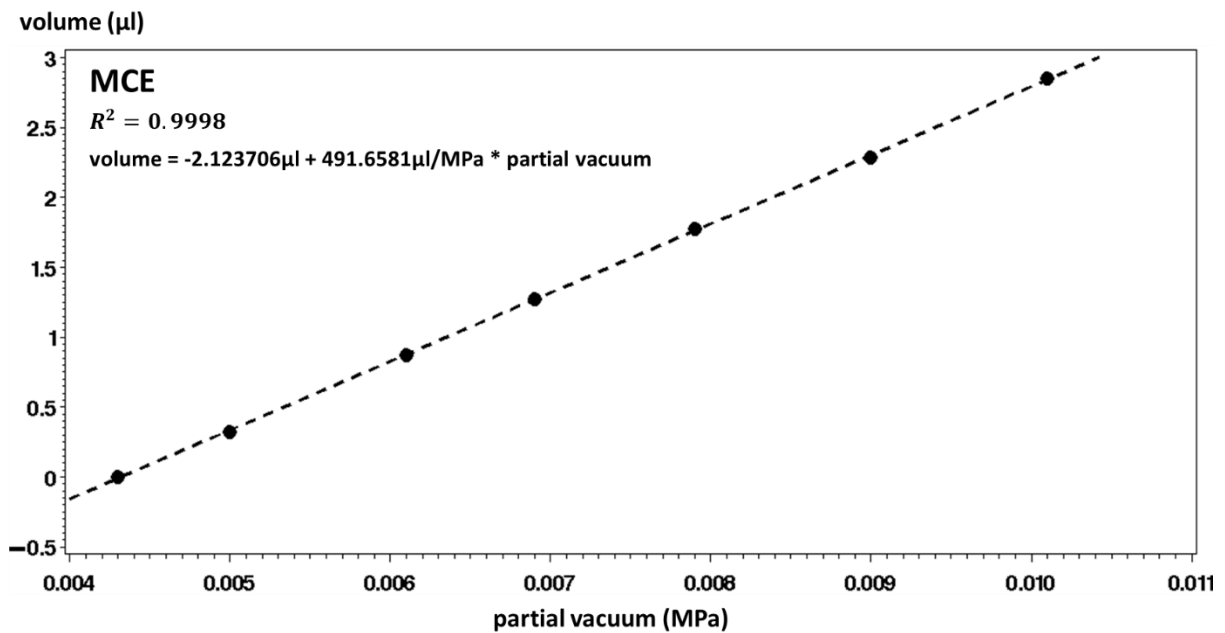

Figure 18: MCE membrane, regression between indicated volume and partial vacuum.

material	slope (µl/MPa)	standard error (µl/MPa)	R^2
unknown	205	7.81	0.991
Polyethersulfone	199	11.5	0.984
MCE	492	2.70	1.000
Nylon	341	21.5	0.982

Table 10: Slope, accompanied standard error and R^2 for the linear regression between volume and partial vacuum for the unknown membrane type and the three commercially available types.

Figure 19 shows the long-term stability experiment for indicated volume at a constant partial vacuum of about 0.006MPa of the unknown membrane type. The more stable the indicated volume over time, the higher the suitability for application in the described quantitative measurement system. Stability of indicated volume can be approximated by the slope of the regression line fitted to the volume data over time. In the experiments, data recording was started when the indicated volume appeared constant again after the alteration in partial vacuum to the desired value of 0.006MPa. Table 11 shows that the magnitude of the slopes from the unknown type, Polyethersulfone as well as MCE have the same magnitude. But considering their standard errors, their might be considerable differences between them. Again, this is only the outcome of one repetition for each type and more data is needed to draw a conclusion. Lastly, we can note that the slope of Nylon was one magnitude smaller than the others' which suggests that Nylon could have more positive properties with respect to long-term stability than the other membrane types.
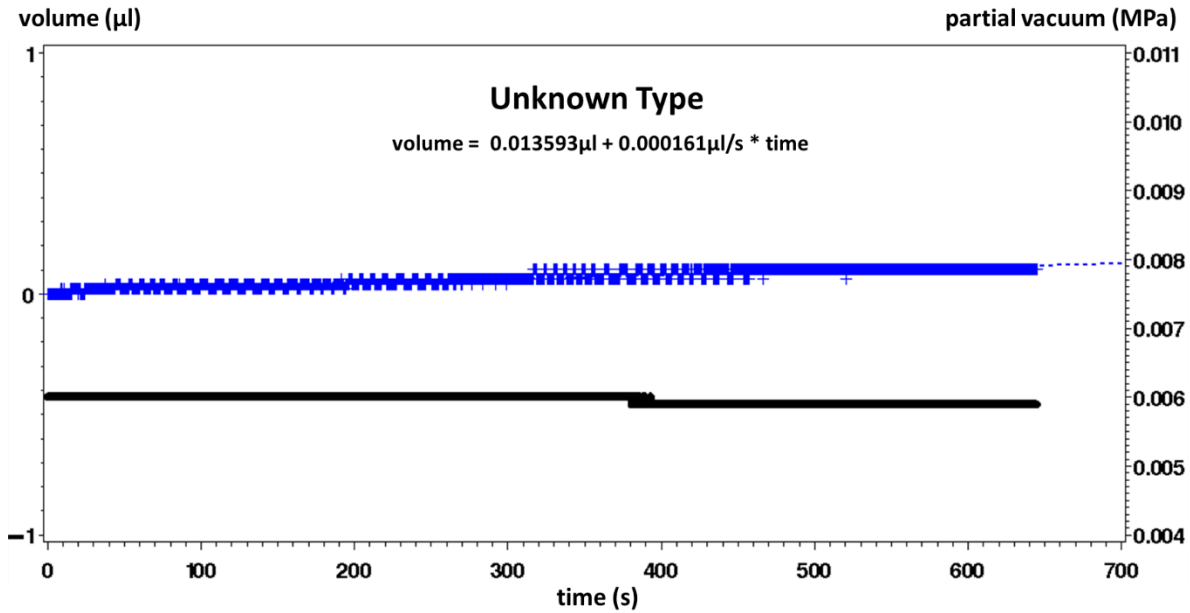
Figure 19: Unknown type, stability of indicated volume (blue) at a constant partial vacuum (black) of about 0.006MPa.

material	slope (µl/s)	standard error (µl/s)
unknown	1.61E-04	1.18E-06
Polyethersulfone	1.56E-04	1.77E-06
MCE	2.96E-04	3.07E-06
Nylon	3.30E-05	9.18E-07

Table 11: Slope and accompanied standard error of the regression lines for volume over time from the long-term stability experiments. Data is shown for the unknown membrane type as well as for the three commercially available types.

To assess how fast the membranes respond to water application, droplets of 1µl were manually applied on the membranes with a microliter syringe. Each droplet application represents a step change in indicated volume. The steps could be approximated nicely with a single exponential function (e.g., figure 20). We decided to use the functions' half times as the indicator for responsiveness. Small half times illustrate high conductivity and thereby high responsiveness, which is favorable for the purpose to absorb efflux on the petiole as fast and as completely as possible. Table 12 shows an ANOVA and subsequent Tukey Test for the half times of the single exponential functions for each membrane type. We can see that

the membrane's type had a significant influence on the half times as well as Nylon having significantly higher half times than the other types, whose values are not significantly different from each other.
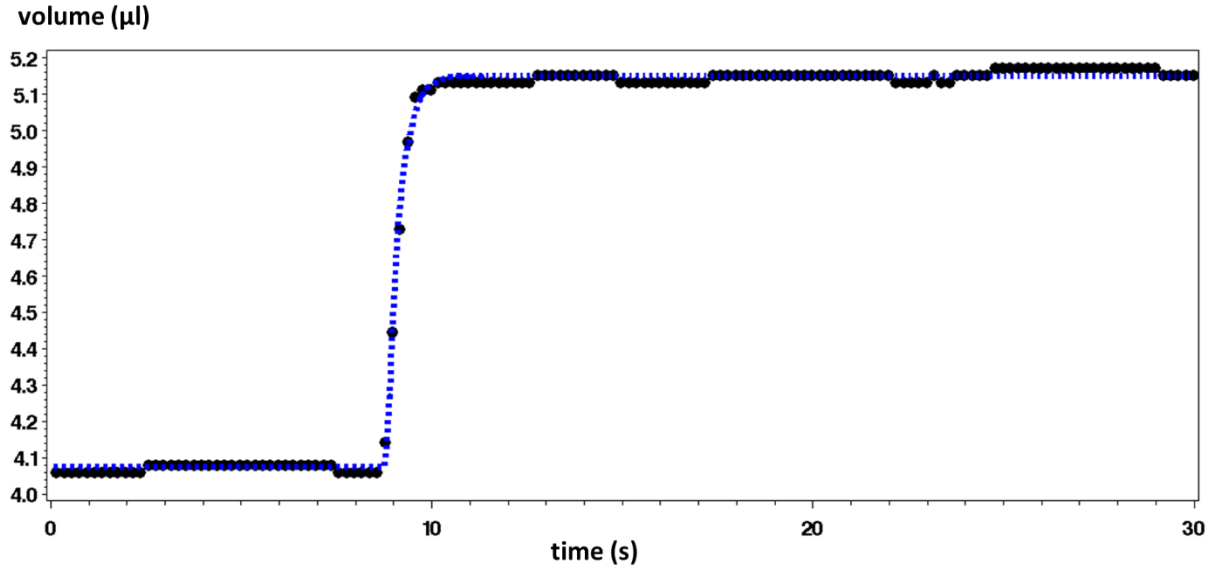

Figure 20: Indicated volume (black) for the application of a 1µl droplet on an MCE membrane. The blue, dotted line shows the approximation of the volume change with a single exponential function.

ANOVA | Test membranes Dependent variable: Half time			
source	**DF**	**F-value**	**Pr > F**
membrane type	3	96.29	< 0.0001
Tukey's Test			
membrane type	**mean half time (s)**	**N**	**Tukey grouping**
unknown	0.243	10	A
Polyethersulfone	0.316	9	A
MCE	0.257	10	A
Nylon	0.705	10	B

Table 12: ANOVA for the half times of single exponential functions approximating the response of a 1µl droplet application for the unknown membrane type and the three commercially available types. The analysis includes 10 measurements for the half time for each of the unknown type, MCE and Nylon; for Polyethersulfone we had 9 measurements for the half time.

Finally, it appears as if commercially available alternatives to the unknown membrane type, that we used for the quantitative measurement system, exist. However, more data is needed to draw a conclusion about the best alternative.

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