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A simple method for measuring apoplast hydration and collecting apoplast contents

Short title not to exceed 50 characters and spaces:

An easy leaf apoplast hydration calculation method

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Article title:

A simple method for measuring apoplast hydration and collecting apoplast contents.

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One sentence summary (200 characters max):

A simple method allows calculation of apoplast hydration and efficient extraction of apoplast contents from maize seedling leaves.

List of Author Contributions:

I.G. and D.M. designed the experiments and analyzed data; I.G., L.G., and W.Z. performed the experiments; D.M and A.A. supervised the experiments; and I.G. wrote the paper with contributions from all the authors.

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Abstract:

The plant leaf apoplast is a dynamic environment subject to a variety of both internal and external stimuli. In addition to being a conduit for water vapor and gas exchange involved in transpiration and photosynthesis, the apoplast also accumulates many nutrients transported from the soil as well as those produced through photosynthesis. The internal leaf also provides a protective environment for endophytic and pathogenic microbes alike. Given the diverse array of physiological processes occurring in the apoplast, it is expedient to develop methods to study its contents. Many established methods rely on vacuum infiltration of an apoplast wash solution followed by centrifugation. In this study, we describe a refined method optimized for maize (*Zea mays*) seedling leaves, which not only provides a simple procedure for obtaining apoplast fluid, but also allows direct calculation of apoplast hydration at the time of harvest for every sample. In addition, we describe an abbreviated method for estimating apoplast hydration if the full apoplast extraction is not necessary. Finally, we show the applicability of this optimized apoplast extraction procedure for plants infected with the maize pathogen *Pantoea stewartii* subsp. *stewartii*, including the efficient isolation of bacteria previously residing in the apoplast. The approaches to establishing this method should make it generally applicable to other types of plants.

Introduction:

The leaf apoplast plays key physiological roles in plants. Leaves consist of an outer epidermal cell layer that confines the inner mesophyll and vasculature tissues. Far from a solid mass of cells, the interior of the leaf has extracellular spaces, collectively known as the apoplast, that are filled with air, liquid, and cell wall material (Sattelmacher, 2000). This open

region of the leaf plays a vital role in the day-to-day physiology of the plant, including transpiration and photosynthesis. Water transport to the shoot is maintained by transpiration; thus, water originating at the roots ultimately arrives in the apoplast prior to water vapor release through stomata (Sattelmacher, 2000; Lawson et al., 2014). Stomata also facilitate the introduction of environmental carbon dioxide into the internal leaf, where it is then consumed during photosynthesis (Lawson et al., 2014). The apoplast further acts as a conduit in the transport of photosynthetically derived sucrose during the phloem-loading process (Geiger et al., 1974; Giaquinta, 1977; Zhang and Turgeon, 2018). Amino acid trafficking to the phloem also requires apoplastic transport (Koch et al., 2003; Lalonde et al., 2003; Zhang and Turgeon, 2018). Finally, specialized metabolism is closely linked with the apoplast during the biosynthesis of cell wall material, especially the deposition of lignins (Liu et al., 2018).

Abiotic stresses such as drought, soil contamination, and light quality can all affect the physiological processes occurring in the leaf apoplast. Decreasing soil moisture has been shown to increase the pH in tomato leaf apoplast (Jia and Davies, 2007; Geilfus, 2017), which in turn can impact the activity of apoplast-exposed, pH-sensitive transporters (Rottmann et al., 2018). The elemental composition of soil also has a direct impact on the leaf apoplast. Calcium, an essential plant nutrient, is absorbed by the roots and deposited into the apoplast prior to being transported into leaf cells (Wang et al., 2017). Silicon, one of the most abundant elements in the Earth's crust, is also deposited in the apoplast, where it is known to confer some nutrient value as well as play a defensive role against invading microbes (Wang et al., 2013; Wang et al., 2017; Coskun et al., 2018; Rasoolizadeh et al., 2018). Silicon can also alleviate the toxicity of excess manganese, another plant nutrient that accumulates in the apoplast (Führs et al., 2009).

89 Physiological roles aside, the apoplast also serves as a niche for endophytic microbes.
90 These organisms, which tend to be present at low levels, maintain a presence that can actually
91 benefit the plant, much as soil microbes offer benefits in the rhizosphere (Kandel et al., 2017).
92 These populations are kept at bay through the plant immune system, including the ability of the
93 plant to control water availability in the apoplast (Xin et al., 2016; Wright and Beattie, 2004).
94 However, foliar pathogens overcome plant defenses to aggressively colonize the leaf apoplast,
95 proliferate to high levels, and cause disease (Jones and Dangl, 2006; Liu et al., 2017). Many
96 foliar pathogens elicit water-soaking in susceptible plants, during which the apoplast
97 accumulates a higher than normal aqueous content that causes the leaf to appear translucent
98 (Asselin et al., 2015; Xin et al., 2016; Schwartz et al., 2017). Plants and their pathogens also
99 compete in various ways to control the availability of sugar in the apoplast, including regulation
100 of host-derived cell wall invertases and pathogen-derived endoglucanase, pathogen-induced
101 transcription of host SWEET family sugar transporters, and host-defense-induced activity of
102 sucrose uptake transporters (Bonfig, et al., 2010; Yamada, et.al., 2016; Veillet, et al., 2016; Cox,
103 et al., 2017). Thus, apoplast-localized events, including the availability of water and nutrients,
104 as well as the mobilization of antimicrobial compounds and materials for cell wall
105 reinforcement, are major determinants of susceptibility versus resistance in plant-bacteria
106 interactions (Kwon, et al., 2008; Beattie, 2011; Xin, et al., 2016; Naseem, et al., 2017).

107 There have been a number of publications detailing centrifugation-based methods for
108 extracting apoplast fluid from plant leaves (Lohaus et al., 2001; Witzel et al., 2011; Joosten,
109 2012; Nouchi et al., 2012; O'Leary et al., 2014). Depending on the plant species and the
110 downstream goal of the apoplast extraction, specific details of the methods vary significantly.

For example, some studies focus on the effects of nutrient deficiencies on the apoplast (López-Millán et al., 2000), while others optimize methods for studies with plant pathogens (Rico and Preston, 2008; Floerl et al., 2012; O'Leary et al., 2016; Schwartz et al., 2017). In this study, we improve upon several current apoplast extraction methods and optimize the procedure for maize (*Zea mays*) seedling leaves. Furthermore, we describe how to calculate leaf apoplast hydration without using dye or oil treatments that are usually included for representative samples. A simplified method for estimating apoplast hydration is also included for when isolation of apoplast contents is not needed. To illustrate the usefulness of this infiltration-centrifugation method, we describe apoplast hydration as a response to the maize pathogen *Pantoea stewartii* subsp. *stewartii* (*Pnss*) and demonstrate efficient isolation of the bacteria along with the apoplast contents.

Results:

General procedures of the apoplast extraction method

The apoplast extraction in this study targets the first true leaf tips of maize seedlings. Due to their small size, a pool of eight 4- to 5-cm-long leaf tips are harvested for each sample and processed to extract their apoplast contents, as shown in Figure 1. After recording the bulked leaves' initial weight (IW) and/or leaf area, they were placed in a 60cc syringe barrel containing the apoplast wash solution. Repeated pull-and-release vacuum cycles with the syringe facilitated the wash solution entrance into the apoplast. Once maximally saturated, the leaves were removed from the wash solution and then gently and thoroughly wiped dry prior to determining the after-infiltration weight (AIW). The leaves were then positioned on a 5x10-cm piece of Parafilm and carefully wrapped around a 1-mL pipet tip for structure. A second piece of Parafilm was added to hold the bundle together and suspended above the bottom of a 15-mL conical tube. This tube, with leaf tips down, was then centrifuged at 2,500 x g for 10 minutes at 4°C. Leaves were weighed post centrifugation to obtain the after-spin weight (ASW). The apoplast liquid released from the leaves, along with any solids (*i.e.* bacteria), was then gently resuspended, transferred to a 1.5-mL microcentrifuge tube and centrifuged at 2320 x g for 5 minutes at 4°C. The supernatant was then transferred to a fresh tube for subsequent analysis.

Evaluation of apoplast extraction efficiency and leaf cellular integrity after the procedure

To verify that the described extraction method was not introducing symplast contamination from ruptured cells, we performed three check experiments of the extraction

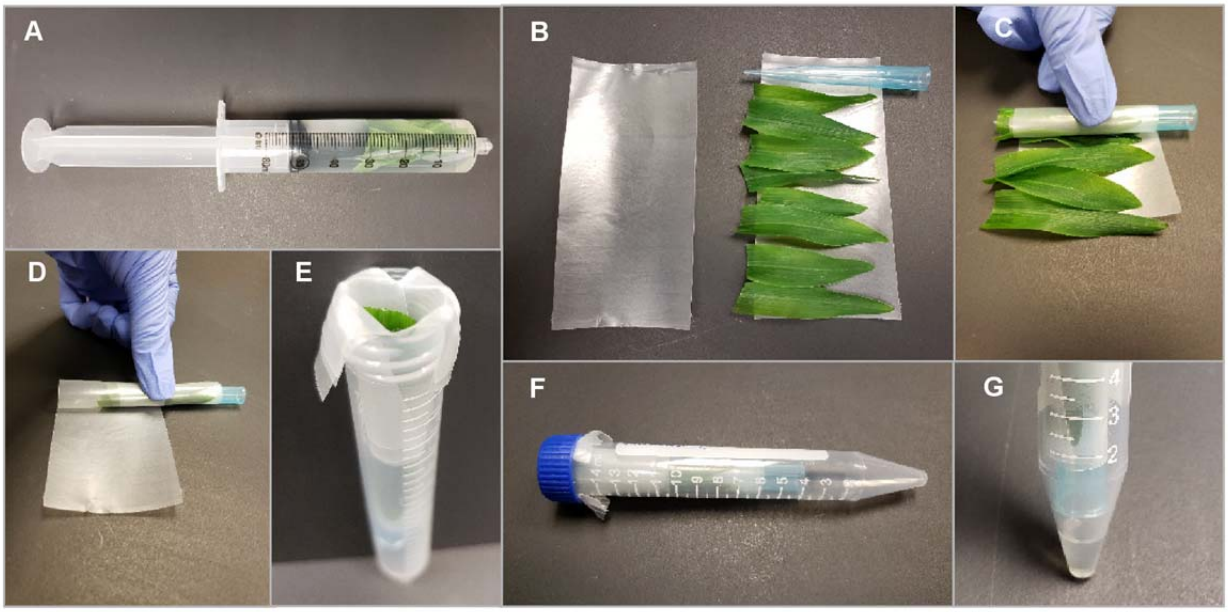

Figure 1. General procedure of maize leaf apoplast extraction. (A) First-true-leaf tips are harvested and syringe-infiltrated with the apoplast wash fluid. (B) The fully saturated leaves are wiped dry on their surface and placed on a 5x10 cm piece of Parafilm. A 1mL pipet tip is oriented in the opposite orientation as the leaves. (C) Keeping the leaves snug against the pipet tip, they are carefully wrapped into a cylinder. (D) To keep the bundle assembled, a second piece of Parafilm is wrapped around the leaves before inserting the bundle into a 15mL conical tube (E). The Parafilm is folded over the top of the tube before replacing the cap (F). The tube is then spun at 2,500 x g for 10 minutes at 4°C. After this step, the aqueous apoplast contents can be recovered separately from the intact leaf bundle (G). Although the leaf bundle often slides downwards during centrifugation, the size of the pipet tip ensures the bundle remains elevated above the liquid extract.

process. The first, shown in Figure 2A, was to determine apoplast wash solutions that maintain cellular integrity. Water has been used as an apoplast wash solution in many studies (Lohaus et al., 2001; Witzel et al., 2011; Joosten, 2012; O'Leary et al., 2014) and was therefore not expected to cause any significant loss of cellular integrity. For applications intended to analyze

148 metabolites from within the apoplast, inclusion of methanol in the apoplast wash solution can
149 increase solubility. Thus, leaves that were syringe-infiltrated with deionized water, 20%
150 methanol, or 40% methanol were tested for cellular integrity. Following syringe-infiltration
151 with each apoplast wash solution, the leaves were incubated in water for 1 hour prior to

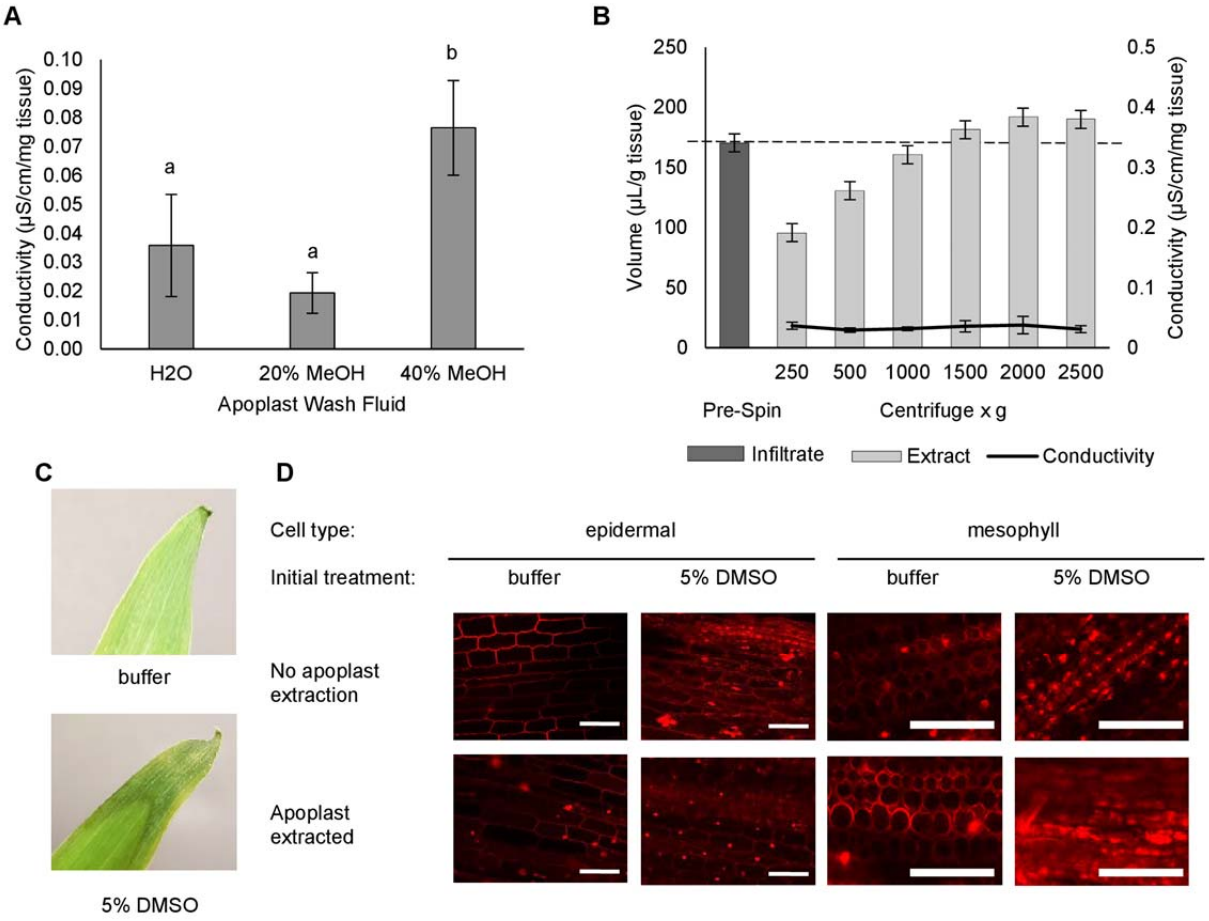

Figure 2. Evaluation of cellular integrity after apoplast extraction. (A) Conductivity of maize seedling leaves syringe-infiltrated with deionized water, 20% methanol, and 40% methanol. Error bars represent standard deviation of three replicates per treatment. Letters signify the significance between treatments, as assessed by one-way ANOVA followed by Tukey's HSD test where $p \leq 0.05$. (B) Impact of centrifugal force on the isolation of apoplast fluid from maize seedling leaves. Infiltrate represents the average volume syringe-infiltrated across all treatments. Conductivity of leaves was assessed after centrifugation. Error bars represent the standard deviation from three replicates for each treatment. (C) Representative photos of maize seedling leaf symptoms at 12 hours after vacuum-infiltration (hai) with buffer or 5% DMSO. (D) Confocal microscopic images of propidium iodide stained epidermal and mesophyll cell layers of maize leaves at 12 hai with buffer or 5% DMSO. Shown are leaves that were not (top images) or were (bottom images) subjected to the apoplast extraction procedure. Two biological replicates, each with two samples per treatment, were analyzed. Scale bars are 100 µm.

measuring the conductivity of the solution. Leaves infiltrated with water or 20% methanol showed similar conductivity, while 40% methanol caused a significant increase in conductivity. Thus, either water or 20% methanol was deemed an appropriate apoplast wash solution. Indeed, metabolite analysis revealed that 20% methanol was suitable for quantification of

amino acids, sugars, organic acids, phosphorylated compounds, and phenolics within a single extract volume of approximately 100 µL from eight maize seedling leaves (unpublished data), at levels comparable to or exceeding those described in (Lohaus et al., 2001).

Next, the impact of centrifugation force on yield and cellular integrity was assessed (Figure 2B). The goal was to identify centrifugation conditions that provide maximal yield of apoplast contents without damaging the integrity of leaf cells. Seedling leaves were syringe-infiltrated with water and apoplast extracts collected by spinning for 10 minutes at a range of centrifugal forces from 250 x g to the instrument maximum at 2,500 x g. The volume of water infiltrated and the volume of liquid extracted were determined by the difference between leaf weight after infiltration and before infiltration or after centrifugation, respectively. It might seem intuitive to directly measure the volume of recovered liquid; however, because some liquid is retained on surfaces of the extraction apparatus, the directly measured volume was consistently less than that calculated from the difference between leaf weight after infiltration and after centrifugation. Thus, the differences in leaf weight are a more reliable measure of extraction. The volume infiltrated per leaf mass prior to the different spins was similar between samples, and the volume extracted per leaf mass increased as expected with the increase in centrifugal force. The extracted liquid exceeded the input water at 1,500 x g and plateaued at 2,000 and 2,500 x g. This indicates full recovery of both the infiltrated water and the preexisting contents of the apoplast. Conductivity measurements of leaves post centrifugation did not differ at any of the tested centrifugal forces and did not differ from those for water-infiltrated, nonextracted leaves (compare conductivity levels in Figure 2B with those in Figure 2A). Furthermore, doubling the centrifugation time at maximum speed did not

significantly increase ion leakage (data not shown). Therefore, to ensure quantitative yield of apoplast contents without any apparent loss of cellular integrity, we used 2,500 x g for 10 minutes as the centrifugation step in this protocol.

Finally, confocal microscopy was used as a further check of leaf cellular integrity after the apoplast extraction process. To assess cellular damage, leaves were stained with 100 µg/mL propidium iodide (PI) for 1 hour to mark the cell walls. PI does not efficiently pass through intact plasma membranes but will stain the nuclei of damaged cells (Jones et al., 2016). It is well documented that dimethylsulfoxide (DMSO) can increase the permeability of lipid bilayers, though few studies have examined this effect in plants (Delmer, 1979; Notman et al., 2006). To generate plants with a permeable plasma membrane to mimic damaged cells, we vacuum-infiltrated maize seedlings with a solution of 0.2% Tween-40 (buffer) or also containing 5% DMSO and waited 12 hours to allow DMSO-induced leaf browning symptoms to develop (Figure 2C). To visualize cellular integrity following these treatments, as well as following the apoplast extraction procedure, confocal microscopy images were collected on the adaxial side of the outermost 1-cm section of leaf tips from these seedlings prior to and post apoplast extraction (Figure 2D).

The status of cells from leaves treated with 5% DMSO showed a different staining pattern compared to the buffer control leaves. While the cell walls of epidermal and guard cells could easily be brought into focus on the buffer-infiltrated plants, the epidermal layer of 5% DMSO-treated leaves showed significant disorganization of cell walls and additional staining of unknown (possibly disrupted) intracellular structures. These disruptions of the epidermal layer are apparent prior to and do not significantly change after the apoplast extraction procedure.

Similarly, DMSO-treated mesophyll cells have nondescript cell walls and an abundance of unidentified intracellular structures both prior to and after apoplast extraction. Thus, PI staining reveals disruptions in maize cell integrity.

Image analysis of non-DMSO-treated leaves revealed that the well-organized PI-staining patterns of cell walls in round mesophyll cells and files of epidermal cells are unperturbed by the apoplast extraction procedure. This indicates that overall cellular integrity remains largely intact following the procedure. The PI staining of intracellular structures (presumably nuclei) is unchanged in mesophyll cells, which are the most abundant cell type, and increases only modestly in epidermal cells. Indeed, while DMSO-treated leaves displayed significant staining of epidermal nuclei before and after the apoplast extraction procedure, buffer-treated plants displayed only modest levels of stained nuclei. Analysis of several images over two replicates revealed that the background level of stained nuclei in buffer-treated leaves was 3.6-7.7% initially and increased to 9.1-11.6% following apoplast extraction (ranges from independent samples with 420 and 173 total cells counted for each analysis, respectively). Thus, the apoplast extraction procedure does not disrupt the ability of the majority of maize cells to exclude PI. Collectively, the data in this section indicate that the apoplast isolation procedure keeps cells of the maize seedling leaves largely intact.

Calculation of apoplast hydration

Understanding the extent to which the apoplast is filled with liquid, which we termed apoplast hydration, is significant to biotic stress and will likely provide useful information in the study of other developmental processes and stress responses in plants. An important aspect of

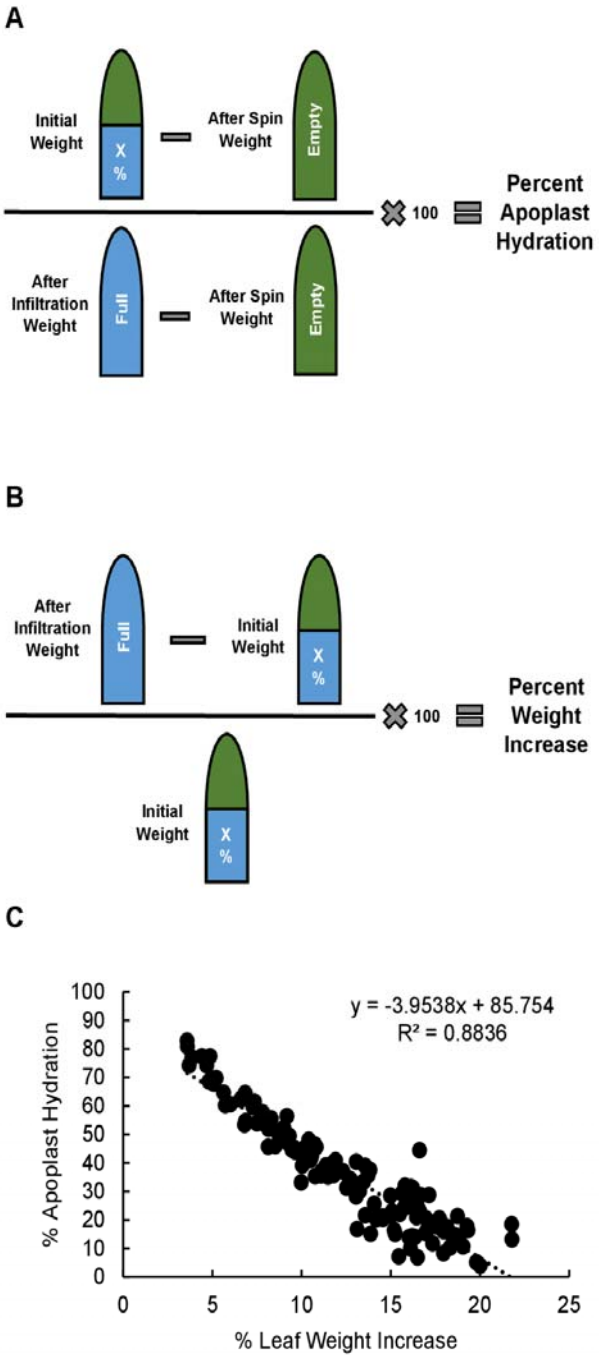

Figure 3. Apoplast hydration calculation. (A) Apoplast hydration can be determined using leaf weights measured during the apoplast extraction procedure. (B) Apoplast hydration also determines the percent weight increase of leaves after the syringe-infiltration step. (C) Linear correlation between apoplast hydration calculated as in (A) and percent weight increase of syringe infiltrated leaves calculated as in (B), where n=130.

221 the method we have developed is that it permits the direct calculation of apoplast hydration

prior to the initiation of the procedure. Specifically, apoplast hydration can be calculated from the leaf weights measured before and after the syringe-infiltration of apoplast wash solution, as well as after centrifugation (Figure 3A). At the time of collection, the apoplast has an unknown level of hydration. Based on our consistent results in Figure 2B, the apoplast is assumed to be fully hydrated after syringe infiltration with the apoplast wash solution and completely evacuated post centrifugation. Therefore, the formula (IW-ASW)/(AIW-ASW) represents the ratio of the original apoplast liquid volume and the overall capacity of the apoplast. It is imperative for experiments across plant types, age, and/or growth conditions that the extraction centrifugal force and duration (as described in Figure 2B) be optimized to ensure accurate apoplast hydration calculation using this formula.

For studies that are solely interested in determining apoplast hydration, it is cumbersome to repeatedly complete all steps of the apoplast extractions outlined above. Instead, after establishing the assay for a given system, an abbreviated method can be performed that provides a close estimate of apoplast hydration. This method uses the leaf weights before and after syringe-infiltration of the apoplast wash solution to calculate the percentage of weight increase of the leaves during this step (Figure 3B). Plotting the percentage of weight increase against the apoplast hydration yields a clear linear correlation (Figure 3C). The equation for this line, which would need to be independently determined for each particular system of study, can solve for apoplast hydration using only the percentage of weight increase. In our case, the equation was generated from 130 apoplast extractions from leaves that were previously untreated or were at various times after vacuum-infiltration of buffer or *Pnss*. The values generated using the estimated method did not differ significantly

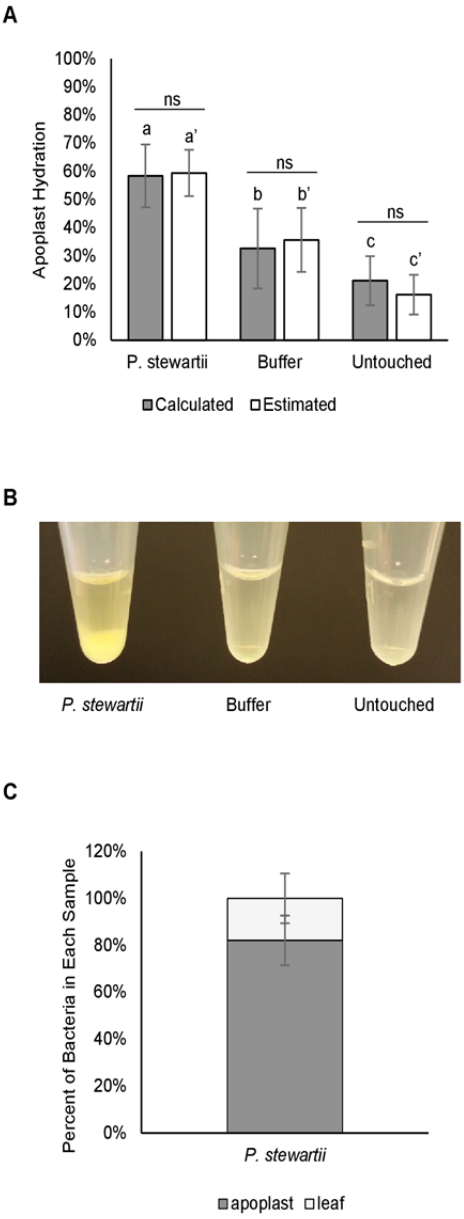

Figure 4. Application of the apoplast extraction method with a maize pathogen. (A) Apoplast hydration of maize leaves 7hai with wild-type *P. stewartii* or buffer, or leaves that were not previously infiltrated. Hydration determined with the calculated vs estimated methods yield non-significantly different values across treatments that are themselves significantly different. Significance between methods was assessed via t-test where p≤0.05, and significance between treatments was assessed via one-way ANOVA followed by Tukey's HSD test where p≤0.05. (B) Representative photos of apoplast extracts isolated from *P. stewartii*- or buffer-infiltrated plants at 12 hai compared to non-infiltrated plants. Note the large bacterial pellet in the *P. stewartii* extract. (C) Distribution of *P. stewartii* between the apoplast extract and the corresponding leaf tissue following apoplast extraction. Error bars represent standard deviation from two biological replicates where n=4.

244 from those utilizing the full apoplast extraction method, and differences in hydration also can

be separated on a by-treatment basis with both methods (Figure 4A).

Application: Maize infection with a bacterial pathogen

Many economically important plant pathogens colonize the leaf apoplast at some point in the infection cycle, and a characteristic symptom of apoplast colonization is water-soaking. *Pnss*, which is the causal agent of Stewart's wilt disease in maize, produces strong water-soaking symptoms early after its colonization of the maize apoplast. This apoplast extraction method has facilitated the quantification of this symptom in infected and control plants (Figure 4A). While apoplast hydration of untreated plants was ~20%, the apoplast hydration of buffer-infiltrated and infected plants was ~30% and ~60%, respectively, at the presymptomatic time point of 7 hours after infiltration (hai). Notably, each of these three levels of apoplast hydration differed significantly, whether calculated by the full or the abbreviated (percentage of weight increase) method. Also, the percentage of increase calculated by each of these methods did not differ significantly for any of the three sample types.

The isolation of microbes from an infected leaf is potentially of great use. The apoplast extracts obtained from *P. stewartii*-infected plants contain a visible bacterial fraction (Figure 4B). To determine the efficiency of pathogen removal from the leaf during the extraction procedure, *P. stewartii* levels were assessed at 12 hai in both the apoplast extract and the remaining leaf tissue. From two biological replicates (n=4 for both isolated apoplast and remaining leaf samples), it was determined that 82% of the viable bacteria were present in the apoplast extract (Figure 4C). Thus, for infection systems where apoplast-localized microbes are efficiently isolated along with the apoplast fluid, this method will also be useful for subsequent

analysis of those microbes immediately following their isolation from the apoplast. We anticipate this method will be particularly useful for bacterial gene expression studies, as apoplast bacteria could be centrifuged directly into an RNA-protecting solution, thus "freezing" the bacterial transcriptome while effectively separating the bacteria from RNA-rich plant tissues.

Discussion and Conclusion:

The apoplast, while a physiologically important compartment of the plant leaf, is not often studied due to the difficulty of examining its contents without disrupting the surrounding tissue. One method that has facilitated study of the apoplast is the infiltration-centrifugation technique. While extraction of apoplast contents can be limited by the properties of the apoplast wash solution, this general method has nonetheless proven useful for the study of proteins, metabolites, and microorganisms localized in the apoplast.

To assess cytosolic contamination of apoplast extracts, activity of cellular enzymes such as malate dehydrogenase or glucose-6-phosphate dehydrogenase is often determined on a portion of the extract (Rico and Preston, 2008; O'Leary et al., 2016). Because of our small sample size (100 µL or less per eight-leaf extraction), we used more sensitive, nonenzymatic tests to confirm that cellular integrity was not disrupted by the apoplast isolation procedure. Conductivity of ions as well as confocal microscopy of propidium iodide-stained cells indicate that we can obtain high-purity apoplast extracts from maize seedling leaves using this optimized infiltration-centrifugation technique. These tests were further confirmed by metabolite analysis, which revealed that apoplasts from untreated leaves contained less than 1% of the total leaf phosphorylated compounds (unpublished data).

Another hallmark of the infiltration-centrifugation technique has been to determine the amount of liquid and/or air space in the apoplast. For example, the volume of liquid in the apoplast has been calculated by infiltrating the apoplast with a solution containing indigo carmine and comparing the extract's absorbance at 610 nm to a standard curve (Solomon and Oliver, 2001; O'Leary et al., 2016). However, potential technical issues exist with this method for some systems depending on the amount of silicon accumulation in the apoplast of the sampled leaf, which is especially common in monocots (Guerriero, et al., 2016). Indigo carmine, which is deep blue, can be cleaved into colorless products in the presence of silicon dioxide (silica) (Cao et al., 2016). This reaction ultimately dilutes the color of the indigo carmine, which in this application would suggest artificially high leaf apoplast hydration. Indeed, in our system with maize seedlings, the indigo carmine assay predicted only a 50% extraction efficiency of apoplast liquid contrary to our conclusive results in Figure 2B. As another method example, the nonaqueous air portion of the apoplast was determined by infiltrating the leaf with silicone fluid (Solomon and Oliver, 2001). In this study, we provide a method that directly measures both apoplast liquid volume and air space – and thus apoplast hydration – using calculations derived from leaf weights before, during, and after the procedure. As a further advantage, rather than representative samples, our method simply determines the apoplast hydration of every sample.

Utilizing this apoplast extraction method, we quantified the water-soaking response of plants infected with the bacterial pathogen *Pnss*. In the course of extracting the aqueous apoplast contents, we recovered a significant portion of apoplast-localized pathogen. The ease with which apoplast hydration can be assessed, as well as the ability to recover microbes from

the apoplast, will facilitate further study on many plant stress responses, including plant-pathogen interactions.

Materials and Methods:

Maize growth conditions

B73 maize (*Zea mays*) seedlings were grown in a Conviron growth chamber set to 30ºC with an 18-hour-light /6-hour-dark cycle. Ten to twelve seeds were planted per round 4-inch pot with MetroMix soil and watered every 36-48 hours or as needed. One to four hours prior to vacuum-infiltration treatments, which are independent of the apoplast isolation procedure, plants were watered and humidity elevated to > 65%.

Bacteria growth conditions

Pantoea stewartii subsp. *stewartii* (*Pnss*) wild-type strain DC283 (Coplin et al., 1986) was grown in LB broth with nalidixic acid (20 μg/mL) selection.

Vacuum infiltration of maize seedlings

Six-day-old B73 maize seedlings were vacuum-infiltrated with buffer or bacterial inoculum as described in (Asselin et al., 2015). Briefly, plants were inverted into a beaker with 300 mL 10 mM KPO_4 buffer (control), *Pnss* at 10^9 CFU/mL, or 5% DMSO (Fisher Chemical D128-500), each containing 0.2% Tween-40 to facilitate infiltration. Using a Nalgene vacuum chamber, up to three pots at a time were subjected to a vacuum of 500 mmHg for 5 minutes, followed by vacuum release. The vacuum treatment was repeated two more times for a total of 15 minutes. Plants were then allowed to air dry for 10-15 minutes until their return to the growth chamber with relative humidity at 65-70%.

Confocal microscopy

Maize seedlings were vacuum-infiltrated with buffer or 5% DMSO as described above. At 12 hai, 1-cm segments of first-true-leaf tips were collected and stained in 100 µg/mL propidium iodide (Invitrogen #P3566 diluted in ddH_2O) for 1 hour on ice and protected from light. With the adaxial side up, each leaf tip was mounted in water and sealed with a #1.5 coverslip and nail polish. Using a Nikon A1+ confocal microscope with 20x objective (numerical aperture 0.75) and 1x confocal zoom, PI-stained cells were visualized by exciting leaves with a 561-nm laser and collecting emitted fluorescence at 605 nm. Images were acquired with Nikon NIS-Elements v4.20 software and further processed in Microsoft PowerPoint. For stained nuclei quantification, only cells with completely visible boundaries were counted.

Apoplast and *in planta* bacteria enumeration

Six-day-old seedlings were vacuum infiltrated as described above with buffer or *Pnss*. At 12 hours after infiltration, eight first true leaf tips were harvested from each treatment, leaf margins traced onto paper for subsequent determination of total leaf area, and apoplast extracted using sterile ddH_2O. After apoplast extraction by centrifugation at 4ºC for 10 minutes at 2,500 x g, leaves were placed on ice and apoplast extracts were resuspended and transferred to 1.5-mL microcentrifuge tubes to repellet the bacteria. Photos of apoplasts isolated from leaves infiltrated with WT bacteria or buffer and from untouched leaves (Figure 4B) were then taken for comparison. Next, the apoplast with WT bacteria was resuspended in cold ddH_2O, serially diluted in septuplicate, and plated on LB solid media with nalidixic acid selection. Two 1-cm disks were removed from each leaf with a cork borer, placed in 1 mL cold ddH_2O, homogenized with 5-mm glass beads in a Qiagen TissueLyser for 1 minute at 30 Hz, serial diluted in triplicate, and plated on LB solid media with nalidixic acid selection. Plates were

incubated for 48 hours at room temperature before determining colony counts. Colony counts from leaf disc samples were converted to total colonies per sample based on the total area of the leaves, which was calculated by processing scanned images of the leaf traces with ImageJ software (https://imagej.nih.gov/ij/).

Conductivity

To assess conductivity from leaves before or after centrifugation, leaves were submerged in 30 mL ddH_2O for 1 hour with occasional gentle agitation. Leaves were then carefully removed and conductivity of the water measured with a WTW Cond 330i meter and TetraCon 325 probe. Blank water conductivity values were subtracted and then the resulting values were normalized to the initial leaf weight prior to syringe infiltration. Averages were obtained from three independent measurements.

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Figure legends

Figure 1. General procedure of maize leaf apoplast extraction. (A) First-true-leaf tips are harvested and syringe-infiltrated with the apoplast wash fluid. (B) The fully saturated leaves are wiped dry on their surface and placed on a 5x10 cm piece of Parafilm. A 1mL pipet tip is oriented in the opposite orientation as the leaves. (C) Keeping the leaves snug against the pipet tip, they are carefully wrapped into a cylinder. (D) To keep the bundle assembled, a second piece of Parafilm is wrapped around the leaves before inserting the bundle into a 15mL conical tube (E). The Parafilm is folded over the top of the tube before replacing the cap (F). The tube is then spun at 2,500 x g for 10 minutes at 4°C. After this step, the aqueous apoplast contents can be recovered separately from the intact leaf bundle (G). Although the leaf bundle often slides downwards during centrifugation, the size of the pipet tip ensures the bundle remains elevated above the liquid extract.

Figure 2. Evaluation of cellular integrity after apoplast extraction. (A) Conductivity of maize seedling leaves syringe-infiltrated with deionized water, 20% methanol, and 40% methanol. Error bars represent standard deviation of three replicates per treatment. Letters signify the significance between treatments, as assessed by one-way ANOVA followed by Tukey's HSD test where $p \leq 0.05$. (B) Impact of centrifugal force on the isolation of apoplast fluid from maize seedling leaves. Infiltrate represents the average volume syringe-infiltrated across all treatments. Conductivity of leaves was assessed after centrifugation. Error bars represent the standard deviation from three replicates for each treatment. (C) Representative photos of maize seedling leaf symptoms at 12 hours after vacuum-infiltration (hai) with buffer or 5% DMSO. (D) Confocal microscopic images of propidium iodide stained epidermal and mesophyll

cell layers of maize leaves at 12 hai with buffer or 5% DMSO. Shown are leaves that were not (top images) or were (bottom images) subjected to the apoplast extraction procedure. Two biological replicates, each with two samples per treatment, were analyzed. Scale bars are 100 µm.

Figure 3. Apoplast hydration calculation. (A) Apoplast hydration can be determined using leaf weights measured during the apoplast extraction procedure. (B) Apoplast hydration also determines the percent weight increase of leaves after the syringe-infiltration step. (C) Linear correlation between apoplast hydration calculated as in (A) and percent weight increase of syringe infiltrated leaves calculated as in (B), where n=130.

Figure 4. Application of the apoplast extraction method with a maize pathogen. (A) Apoplast hydration of maize leaves 7hai with wild-type *P. stewartii* or buffer, or leaves that were not previously infiltrated. Hydration determined with the calculated vs estimated methods yield non-significantly different values across treatments that are themselves significantly different. Significance between methods was assessed via t-test where $p \leq 0.05$, and significance between treatments was assessed via one-way ANOVA followed by Tukey's HSD test where $p \leq 0.05$. (B) Representative photos of apoplast extracts isolated from *P. stewartii*- or buffer-infiltrated plants at 12 hai compared to non-infiltrated plants. Note the large bacterial pellet in the *P. stewartii* extract. (C) Distribution of *P. stewartii* between the apoplast extract and the corresponding leaf tissue following apoplast extraction. Error bars represent standard deviation from two biological replicates where n=4.

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