MEASUREMENT OF GAS EXCHANGE ON EXCISED GRAPEVINE LEAVES DOES NOT DIFFER FROM *IN SITU* LEAVES, AND POTENTIALLY SHORTENS SAMPLING TIME

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Measurement of Gas Exchange on Excised Grapevine Leaves Does Not Differ from *In Situ* Leaves, and Potentially Shortens Sampling Time

Suraj Kar [1], Thayne Montague [1,2,*], Antonio Villanueva-Morales [3] and Edward Hellman [4]

[1] Department of Plant and Soil Science, Texas Tech University, Lubbock, TX 79409, USA; surkar@ttu.edu
[2] Texas A&M University AgriLife Research and Extension Center, Lubbock, TX 79403, USA
[3] Department of Statistics, Mathematics and Computing, Forest Sciences Division, Universidad Autónoma Chapingo, Texcoco 56235, Mexico; antoniov28@gmail.com
[4] Department of Plant and Soil Science, Texas Tech University, Fredericksburg, TX 78624, USA; ed.hellman@ttu.edu
* Correspondence: thayne.montague@ttu.edu; Tel.: +1-8068347311

Featured Application: Measuring gas exchange using infra-red gas analyzers enables an enhanced sample size.

Abstract: Use of leaf gas exchange measurement enhances the characterization of growth, yield, physiology, and abiotic stress response in grapevines. Accuracy of a crop response model depends upon sample size, which is often limited due to the prolonged time needed to complete gas exchange measurement using currently available infra-red gas analyzer systems. In this experiment, we measured mid-day gas exchange of excised and *in situ* leaves from field grown wine grape (*Vitis vinifera*) cultivars. Depending upon cultivar, we found measuring gas exchange on excised leaves under a limited time window post excision gives similar accuracy in measurement of gas exchange parameters as *in situ* leaves. A measurement within a minute post leaf excision can give between 96.4 and 99.5% accuracy compared to pre-excision values. When compared to previous field data, we found the leaf excision technique reduced time between consecutive gas exchange measurements by about a third compared to *in situ* leaves (57.52 $\pm$ 0.39 s and 86.96 $\pm$ 0.41 s, for excised and *in situ*, respectively). Therefore, leaf excision may allow a 50% increase in experimental sampling size. This technique could solve the challenge of insufficient sample numbers, often reported by researchers worldwide while studying grapevine leaf gas exchange using portable gas exchange systems under field conditions.

Keywords: CO_2 assimilation rate; stomatal conductance; measurement time; photosynthetic decline curve; infra-red gas analyzer

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1. Introduction

With currently available infrared gas analyzers, such as the LI-6400XT system (LI-COR Biosciences, Lincoln, NE, USA), estimating physiological parameters *in situ* generates accurate data [1]. However, the prolonged time period required to collect each measurement limits the ability of researchers to collect large numbers of data samples [2,3]. Montague and McKenney (2018) [2] reported that moving and positioning the LI-6400XT system for each individual leaf measurement required a relatively long period of time (~1.8 min between two consecutive measurements), which resulted in a relatively restricted sample size (e.g., 33 samples per hour) [1]. Sample size is critical to experimental design, as well as interpretation of results [4,5].

The limited reach of most gas exchange measurement devices into plant canopies, particularly large woody perennial plants, often makes measuring leaf gas exchange cumbersome to implement [6,7]. For instance, due to restricted maneuverability, Kar et al. (2019) [8]

had to move potted plants to access their leaves for leaf gas exchange measurements. Consequently, to measure leaf level gas exchange under field conditions researchers have resorted to modifying devices [9–11] and sampling techniques [7].

The ability of leaf tissue to retain water after a leaf is excised has been previously studied in laboratory conditions in wheat (*Triticum aestivum*) [12], and cotton (*Gossypium hirsutum*) [13]. Furthermore, photosynthetic decline curves post leaf excision have been studied under laboratory conditions in corn (*Zea mays*) [14]. Photosynthetic recovery post water stress has also been studied in grapevines (*Vitis vinifera*) using excised leaves [15]. Due to the ability of a leaf to remain turgid for a period of time after being excised [3,14], photosynthesis for an excised leaf may not substantially decline for a period after excision. This period could be used to increase the number of leaves sampled and measured for gas exchange in experiments conducted in the field or greenhouse.

Previous researchers have excised leaves to study various plant characteristics [16,17]. Excised leaves have been used to study photosynthetic carbon assimilation (A_n) in wheat (*Triticum aestivum*) [18], grapevines (*V. vinifera*) [15], sorghum (*Sorghum bicolor*) [19] and peas (*Pisum sativum*) [20] under laboratory conditions. In addition, McAusland et al. (2019) [21] used leaf chlorophyll fluorescence imaging to perform high throughput phenotyping for photosynthetic and photo-protective parameters in wheat using excised leaves. This method enabled them to study more than 500 samples each day.

To measure leaf gas exchange in grapevines, researchers generally measure photosynthesis on attached leaves [22,23], and move measurement devices from leaf to leaf, and plant to plant. Although several studies have incorporated leaf excision to characterize physiological parameters in various plant species under laboratory conditions [15,18,21], there is still a lack of information on using leaf excision techniques when facing logistical challenges estimating grape leaf gas exchange in field settings. In a two-phase experimental and analytical study, we conducted a field experiment to (1) determine whether measuring gas exchange on excised grape leaves provides an accurate measure of gas exchange parameters, (2) determine whether the leaf excision technique reduces time between consecutive measurements compared to field data from experiments conducted on *in situ* leaves.

2. Materials and Methods

2.1. Field Experiment Testing Accuracy of Gas Exchange Measurement on Excised Grape Leaves

Research comparing gas exchange of excised and *in situ* grape leaves was conducted in 2013 and 2014 at Texas A&M AgriLife Research and Extension Center vineyard in Lubbock, TX (33°41′33″ N; 101°49′17″ W). The vineyard was established in 2004 and has a north–south row orientation with 1.8 m × 3.0 m (vine × row) spacing. Vines were sprawl trained with bilateral cordons (height of 90 cm), and spur pruned to four spurs per cordon and two buds per spur [24]. Soil at the vineyard site was a deep, well drained Olton series fine sandy loam, which has been described previously [25]. Five cultivars were included (Cabernet franc, Cabernet sauvignon, Chardonnay, Grenache, and Tempranillo), which were bench grafted onto 110R rootstocks. All vines used for excised and non-excised gas exchange measurements were well watered, and managed according to viticultural practices common to the region [26].

Leaf gas exchange measurements were made on 1 and 8 August 2013 and 30 May and 3 June 2014. Data collection was conducted on cloudless days. Mid-day (±1 h of solar noon) leaf stomatal conductance (g_s), and net photosynthetic rate (A_n) were measured using two LI-6400XT systems. Each machine was equipped with a 6400-02B red/blue external LED light source, a CO_2 mixer, and a leaf cuvette attached to a tripod. To simulate environmental growing conditions during measurements, light intensity within each LI-6400XT chamber was maintained at ambient levels, which ranged between 1500 and 2000 μmol/m^2/s over the measurement period, representative of a light-saturated photosynthetic state [27]. Chamber CO_2 was sustained at 400 μL/L. Flow rate was set at 500 μmol/s. To measure leaf temperature and ambient relative humidity, the leaf cuvette was clamped to a nearby non-sample leaf prior to and several times during daily measurement periods. Conditions

within each cuvette were then set to closely represent these conditions [28]. In all cases leaf temperature was recorded between 33 and 35 °C. All leaves measured were fully exposed, mature, and selected from positions seven to nine from the shoot tip [29].

Experimental design for each cultivar was a randomized complete block/paired with the two treatments, excised and *in situ*, and 16 blocks. The block factor of the design was represented by the measurement time, with 10 replicate times in 2013 and 6 replicate times in 2014. At each measurement time (replicate), gas exchange of two leaves exposed to full sunlight in close proximity on the same shoot of an individual vine were measured simultaneously. One of the leaves, selected randomly, was excised during the measurement period. At the initiation of the measurement, leaf chamber conditions were set to identical settings. Cuvettes were clamped onto leaves at the same time, and gas exchange parameters were allowed to reach a steady state. Once equilibrium was achieved in each cuvette, an auto program recorded gas exchange measurements every 30 s, and ran for a total of 10 min. After 120 s, the petiole of the leaf randomly assigned to the excised treatment was cut just above its subtending node (a minimum 2.5 cm of petiole remained attached to the leaf). On each measurement date, once the auto program was complete for one set of leaves, LI-6400XT machines were moved to a random vine of another cultivar and the process was replicated. This process was repeated during each measurement date for each cultivar over the course of the two experiment years. Therefore, sixteen simultaneous measurements of leaf gas exchange were recorded for excised and *in situ* leaves for each of the five cultivars.

2.2. Analysis of Leaf Gas Exchange Data from Past Field Experiments

To test whether using excised leaves reduces measurement time in actual field-level gas exchange measurements, data from field experiments conducted over several years were collected on different wine grape cultivars grown at four locations near Lubbock, TX (Table 1). All vines used for gas exchange measurements were well watered and managed according to viticultural practices common for the region [26]. Measurement protocol for *in situ* leaves consisted of moving the LI-6400XT system and tripod from leaf to leaf, and from plant to plant. Once a suitable leaf was found, the cuvette was clamped onto the leaf, and leaf gas exchange was measured until stable levels for A_n and g_s were reached (CV < 10%). Gas exchange data were then logged by the operator. This protocol was used to measure gas exchange from two leaves for each grapevine. Excised leaf gas exchange data were determined in a similar fashion as non-excised leaves, except instead of moving the LI-6400XT system and tripod from leaf to leaf, and plant to plant, the system remained in a location central to three or four plants which were to be measured. The operator then selected a random leaf from a plant and, using a sharp instrument, excised the leaf from the plant (leaving a minimum of 2.5 cm of petiole attached to the leaf). The excised leaf was then taken to the LI-6400XT system, and leaf gas exchange was measured in a similar manner as *in situ* leaves.

Table 1. Cultivars, locations, and field gas exchange data collection dates over the years for experiments involving *in situ* and excised leaves of wine grape (*Vitis vinifera*) cultivars. Under excision treatment, leaves were excised at the base of the petiole and brought to LI-6400XT leaf cuvette. While for *in situ* leaves, the leaf cuvette was clamped onto a leaf attached to the vine.

Treatment	Year	Rootstock	Scion	Location	Collection Date
In situ	2006	Harmony	Carignon, Grenache	Ropesville, TX (33°24′55″ N; 102°9′33″ W)	25 August and 7 September
	2008	5BB, 44-53, 110R, 1103 P 110R	Merlot Grenache, Cabernet sauvignon, Pinot noir, Mourvedre	Texas A&M AgriLife Research and Extension Center vineyard in Lubbock, TX (33°41′33″ N; 101°49′17″ W)	3 June, 10 June, 16 June, 19 June, 26 June, 5 August, 7 August, 14 August, and 21 August
	2009	5BB, 44-53, 110R, 1103 P 110R	Merlot, Grenache, Cabernet sauvignon, Pinot noir, Mourvedre	Texas A&M AgriLife Research and Extension Center vineyard in Lubbock, TX (33°41′33″ N; 101°49′17″ W)	21 May, 26 May, 9 June, 16 June, 1 July, 8 July, 15 July, 21 July, 28 July, 29 July, 6 August, 11 August, 21 August, 27 August, 31 August, and 21 September
	2010	Own Rooted, Riparia, 1103 P, SO4, Freedom, Harmony, 3309C, 420A, 5BB	Cabernet sauvignon	Meadow, TX (33°20′16″ N; 102°12′33″ W)	27 July, 3 August, 17 August, and 24 August
Excised	2016	110R 110R, Freedom, 1103 P	Cabernet sauvignon Grenache	Texas A&M AgriLife Research and Extension Center vineyard in Lubbock, TX (33°41′33″ N; 101°49′17″ W)	13 June and 25 July
	2017	110R 110R, Freedom, 1103 P	Cabernet sauvignon Grenache	Texas A&M AgriLife Research and Extension Center vineyard in Lubbock, TX (33°41′33″ N; 101°49′17″ W)	6 June, 20 July, and 7 August
	2018	Own rooted	Malbec, Pinot gris	Brownfield, TX (33°10′37″ N; 102°16′3″ W)	17 May, 1 June, and 18 June

2.3. Data Analysis

For the field experiment involving excised and non-excised leaves, the SAS procedure GLIMMIX (version 9.4; SAS Institute Inc., Cary, NC, USA) was used to perform statistical analyses of gas exchange data. Net carbon assimilation and stomatal conductance data were analyzed separately for each of five grape cultivars by fitting a repeated measures linear mixed model, with time point being the repeated measures factor. The Akaike information criterion corrected (AICc) produced the appropriate covariance structure for the repeated measures [30], from several alternative candidate models. In all cases, the AICc selected a compound symmetry covariance structure for the repeated measurements (30 s, 60 s, 90 s, . . . , 570 s). Terms for the fixed main effects, the interaction of the factors treatment (*in situ*, excised) and time point of measurement were included in the linear predictor for the mixed model. Year was regarded as a fixed covariate effect and replicate within a year was regarded as a random effect. Comparisons between means were performed through the SAS LSMEANS statement using ADJ = TUKEY option to obtain a Tukey–Kramer multiple comparison adjustment of *p*-values for differences in LS means. In each case, significance was tested at $\alpha = 0.05$. Correlation analysis between A_n and g_s was performed using R v 3.5.1 (R Core Team, 2015). The "ggplot" package in R v. 3.5.1 (R Core Team, 2015) was used to create regression lines and equations. GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) was used to draw figures.

Data analysis for past field experiments consisted of estimating time transpired between measuring gas exchange on one leaf to the next. Measurement time was calculated from original LI-6400XT data sheets. Time differences were estimated for each consecutive measurement under each measurement date using Microsoft Excel (version 2013; Microsoft Corporation, WA, USA). Data outliers (times considered too fast or too slow compared to other data) were removed by arranging data in a scaler order and excluding the slowest and fastest 5% of data from the data set. Gas exchange measurement time efficiency of excised and *in situ* leaves was compared. "Treatment" consisted of time between consecutive measurements in the field. To compare mean duration between measurements in excised and *in situ* treatments an independent unequal variance samples t-test ($\alpha = 0.05$) methodology was implemented using the TTEST procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

3.1. Accuracy of Gas Exchange Measurements on Excised Leaves

Comparing accuracy of gas exchange measurements on field-grown excised and *in situ* leaves indicated a potential 'window' in time where gas exchange in both the treatments remained statistically identical (Figure 1). At the point of excision (120 s), A_n ranged from 14.8 ± 1.1 µmols m^{-2} s^{-1} in Tempranillo to 18.3 ± 0.6 µmols m^{-2} s^{-1} in Cabernet franc, respectively, while g_s ranged from 0.34 ± 0.03 mols m^{-2} s^{-1} in Grenache to 0.22 ± 0.02 mols m^{-2} s^{-1} in Tempranillo, respectively (Figure 1). Under *in situ* treatment, each cultivar maintained stable A_n and g_s throughout the measurement period (Figure 1). Cabernet franc maintained the greatest A_n rates (17.9 ± 0.6–18.7 ± 0.6 µmols m^{-2} s^{-1}) under the *in situ* treatment, while Tempranillo had the lowest A_n rates (14.6 ± 1.1–14.9 ± 1.1 µmols m^{-2} s^{-1}) (Figure 1). Continued gas exchange measurements after leaf excision revealed a decrease in A_n and g_s in all cultivars following a standard sigmoid curve (Figure 1). However, across each cultivar, A_n data suggest even after a minute post excision gas exchange can be recorded at a 96.4 to 99.5% accuracy compared to pre-excision. Cultivars showed differing lengths of time within which gas exchange could be accurately measured (Figure 1). While Grenache data indicate a wide window of 330 s post excision, Tempranillo data reveal a much narrower window of 180 s post excision (Figure 1). Remaining cultivars showed an intermediate time window between 210 and 240 s. A quadratic equation fit ($R^2 > 0.99$) between A_n and g_s post excision across cultivars indicates that closure of stomata directly results in reduction in photosynthetic CO_2 assimilation when leaves were detached from the plant (Figure 2). Although data suggest no significant drop in gas exchange within each

time window, from the point of excision to the end of the window, there is a very apparent numerical decrease in leaf gas exchange rates. Hence, we suggest gas exchange on excised grape leaves be recorded within a maximum of 50% of the length of the time window.

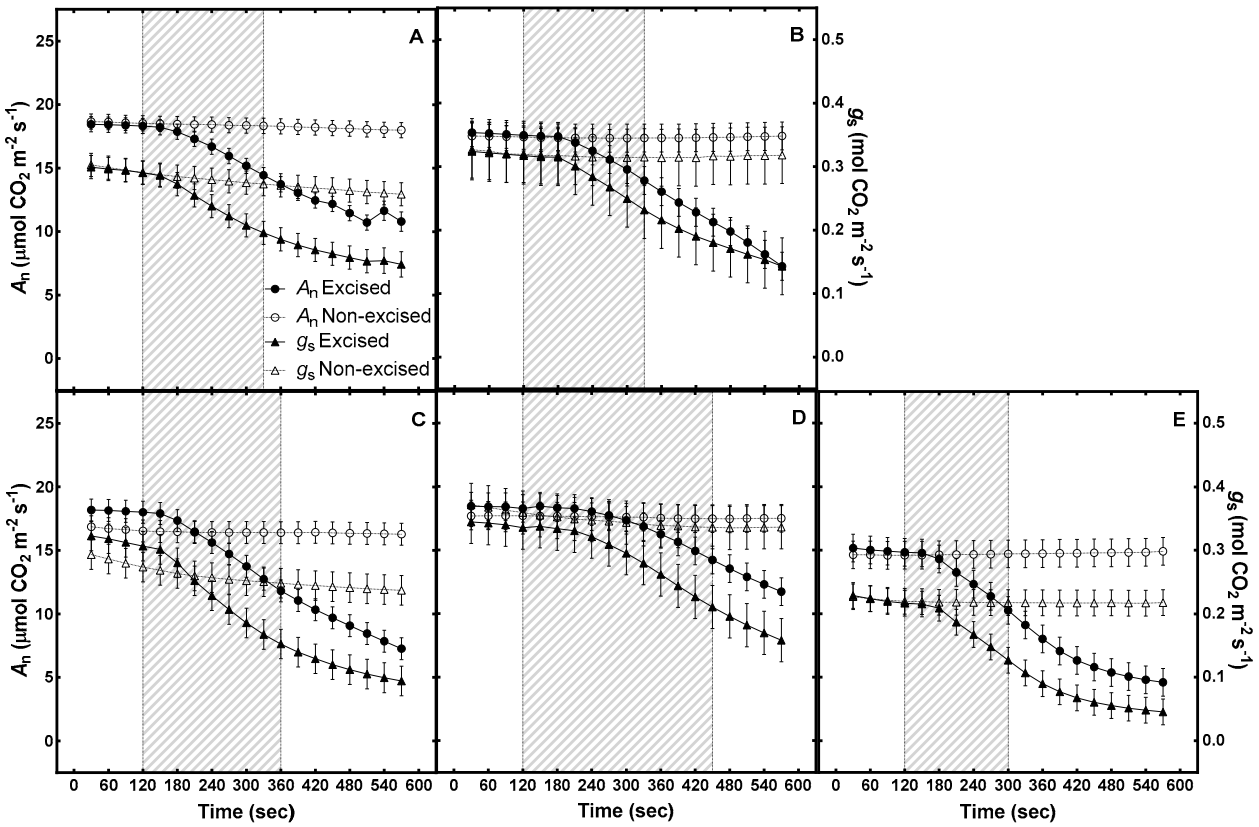

Figure 1. Least square means ± SE for A_n and g_s, measured over time on excised and *in situ* leaves of five grape (*Vitis vinifera*) cultivars: Cabernet franc (**A**), Cabernet sauvignon (**B**), Chardonnay (**C**), Grenache (**D**), and Tempranillo (**E**) over a 10-min time period, plotted on the *x*-axis. Leaf petioles were excised at 120 s in the 'Excised' treatment. Area shaded within each panel indicates a time 'window' in which the gas exchange rate in an excised leaf remains statistically similar with a respective *in situ* leaf. Separation of means was performed using adjusted *p*-values obtained from the Tukey–Kramer multiple comparison procedure ($\alpha = 0.05$). Means were significantly different at the right-hand border of the shaded area ($p < 0.0001$). Each symbol represents the mean of 16 measurements.

The duration of A_n remaining stable post leaf excision has been observed in several ornamental tree species such as Shantung maple (*Acer truncatum*), Mexican redbud (*Cercis canadensis* var. *mexicana*), Texas redbud (*C. canadensis* var. *texensis*), white Texas redbud (*C. canadensis* var. *texensis* cv Alba), Oklahoma redbud (*C. canadensis* var. *texensis* cv Oklahoma), Chinquapin oak (*Quercus muehlenbergii*), and English oak (*Q. robur*) [3]. Relatively stable post excision A_n values observed in every cultivar were likely due to maintenance of water status in leaf tissue [3,12–14]. The rapid drop in A_n past the post excision 'window' as observed in our study, which is consistent with previous findings [3,14], indicates rapid stomata closure to prevent water loss from leaf tissue [13]. Stomatal conductance decreases quickly under rapidly decreasing leaf water status, thus restricting flow of CO_2 into leaf tissues, and resulting in a decrease in A_n [31]. However, CO_2 assimilation may still continue as long as sufficient water is retained by leaf tissue after excision occurs [14]. Other related gas exchange parameters such as transpiration, leaf to air vapor pressure deficit, and leaf internal CO_2 partial pressure, showed similar trends to A_n and g_s (data not shown).

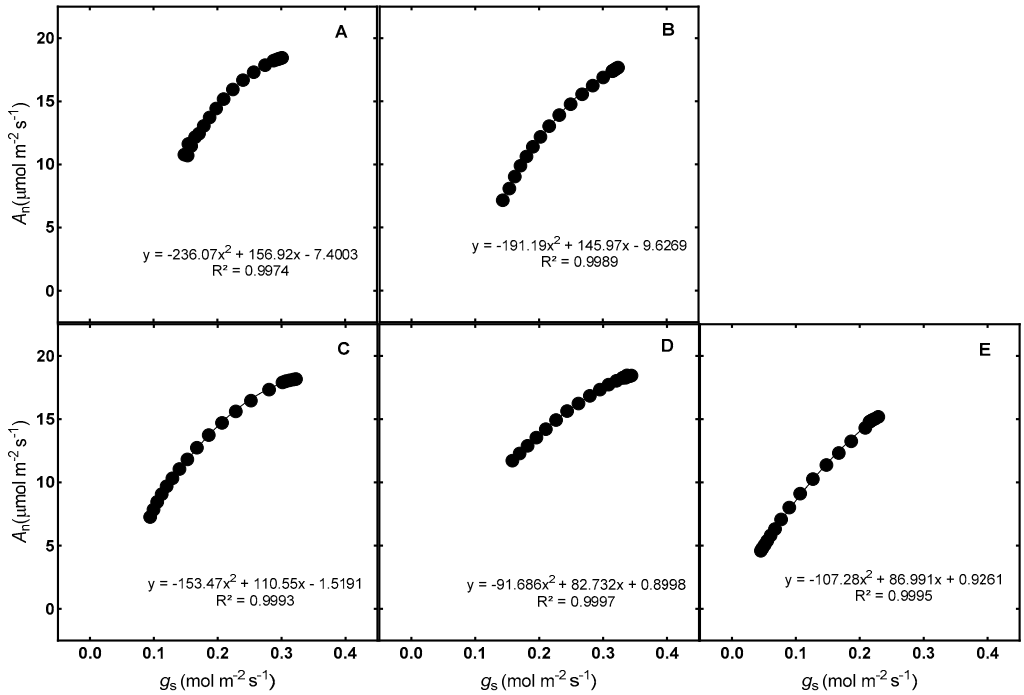

Figure 2. Net CO_2 assimilation (A_n) vs. stomatal conductance (g_s) for five grape (*Vitis vinifera*) cultivars: Cabernet franc (**A**); Cabernet sauvignon (**B**); Chardonnay (**C**); Grenache (**D**); and Tempranillo (**E**) under excision treatment. Lines are the best fit (α = 0.05). Regression equations and R^2 values were shown within each panel. Each symbol represents the mean of 16 measurements.

3.2. Duration between Gas Exchange Measurements of In Situ Leaves

Comparison of gas exchange measurement times of *in situ* and excised grape leaves from field experiments conducted over a number of years gave a clear perspective of sample size and time between consecutive measurements of grapevine gas exchange (Table 2). Gas exchange measured on *in situ* leaves over four years (2006, 2008, 2009, and 2010) on six *V. vinifera* cultivars (Carignon, Merlot, Grenache, Cabernet sauvignon, Pinot noir, and Mourvedre) yielded a total of 2388 sample data points (Table 2). Excised leaves measured over three years (2016, 2017, and 2018) across four cultivars (Cabernet sauvignon, Grenach, Malbec, and Pinot gris) produced 710 data points (Table 2). Statistical analysis revealed about a third less time was required to complete two consecutive measurements on excised leaves (57.52 ± 0.39 s) compared to *in situ* (86.96 ± 0.41 s) (Table 2). Based upon these results, we affirm a significant difference in time between excised and *in situ* treatments ($p < 0.001$) (Table 2). The mean transpired time for the excision treatment was about 30 s faster when compared to mean transpired time for the *in situ* treatment. This implies that on a typical day of gas exchange measurement (±2 h of solar noon) about 250 data points could be recorded using leaf excision technique as opposed to 165 data points using *in situ* leaves. The time saved described in our experiment is situational and depends on experimental design, distance between plots, number of individuals taking measurements, etc. However, leaf excision could be a feasible option in specific experimental conditions such as vineyards, greenhouses, or growth chambers where vines are tightly spaced.

Measurement times observed in our experiment were similar to previous research conducted on ornamental tree species such as Shantung maple (*Acer truncatum*), Mexican redbud (*Cercis canadensis* var. *mexicana*), Texas redbud (*C. canadensis* var. *texensis*), white Texas redbud (*C. canadensis* var. *texensis* cv Alba), Oklahoma redbud (*C. canadensis* var. *texensis* cv Oklahoma), Chinquapin oak (*Quercus muehlenbergii*), and English oak (*Q. robur*) [1–3]. A reduced period between consecutive measurements observed in the excision data compared to *in situ* data indicates an opportunity for researchers to increase the number of sample leaf gas exchange measurements over the same period of time, and thus increase

sample size. Greater sample size is crucial in determining data patterns [32], and developing accurate explanatory models [33]. Leaf excision techniques have previously been used by various researchers to measure leaf gas exchange parameters [7,34,35]. However, these reports did not focus on the potential benefits of increased sample size. Although our current study is comparable to a previous experiment [3] accuracy of the technique will likely depend upon species, experimental design, plant water status, environmental conditions, leaf position, cultivar, etc. In addition, our results should not be considered a benchmark, and representative or comparable to grapevine leaf gas exchange results from past, or future experiments. Thus, as is standard practice, leaf gas exchange levels from a single research experiment should not be directly compared to other experiments. Results should be examined and compared only to data collected and analyzed within each individual experiment. However, if followed, techniques described in this paper will likely give researchers an advantage of high precision with greater speed when estimating grapevine leaf gas exchange.

Table 2. Comparison of time between two consecutive *in situ* gas exchange measurements for *in situ* and excised leaf treatments in numerous wine grape (*Vitis vinifera*) cultivars. Under excision treatment, leaves were excised at the base of the petiole, and brought to the LI-6400XT leaf cuvette. While for *in situ* leaves, the leaf cuvette was clamped onto a leaf attached to the vine.

Treatment	Sample Size (n)	Time between Measurements (s)
In situ leaves [a]	2388	86.96 ± 0.41 [c]
Excised leaves [b]	710	57.52 ± 0.39
95% confidence interval for mean difference between *In situ* and Excised treatments		(−30.56, −28.33)

[a] *In situ* leaf data in collected 2006, 2008, 2009, and 2010 (Merlot, Carignon, Grenache, Cabernet sauvignon, Pinot noir, and Mourvedre). [b] Excised leaf data collected in 2016, 2017, and 2018 (Cabernet sauvignon, Grenache, Malbec, and Pinot gris). [c] Mean ± Standard error values are reported. Mean separation time between two consecutive measurements was conducted using an independent unequal variance samples *t*-test ($\alpha = 0.05$). Time between measurements means were significantly different ($p < 0.0001$).

4. Conclusions

In these experiments, we describe a technique to measure grape leaf gas exchange within a 'window' of time in which observed that A_n and g_s values remain stable and similar to *in situ* leaves. Although this 'window' shows a statistical similarity between excised and *in situ* leaves, experience suggests measurements should be made as quickly as possible post excision to keep quality of data intact. Comparison of the time required to measure gas exchange from excised and *in situ* leaves demonstrates the leaf excision technique reduced time between consecutive measurements. Not only does this novel technique provide an accurate measure (>96.4%) of grapevine leaf gas exchange parameters within a limited window of time, but due to reduced time between consecutive measurements there is a potential to increase the experimental sample size by approximately 50%.

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