

A compact and portable system for simultaneous CO₂ and O₂ measurements of leaf respiratory quotient

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Research Article

Keywords: Leaf respiration, Respiratory quotient, Simultaneous CO₂ and O₂ measurements, Gas exchange, Optical oxygen sensor, Low-cost instrumentation, Infrared gas analyzer (IRGA)

Posted Date: October 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11352844/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Leaf respiratory quotient (RQ), defined as the ratio of CO₂ production to O₂ consumption, provides valuable information on respiratory metabolism, including respiratory substrate utilization and other metabolic processes affecting CO₂/O₂ stoichiometry. However, RQ estimates may be affected by methodological biases when CO₂ and O₂ fluxes are measured independently using different systems and leaf chambers. Here, we developed a compact, portable, and low-cost closed gas-phase system combining a miniaturized Sensirion CO₂ sensor with a PyroScience optical O₂ sensor, enabling simultaneous determination of respiratory CO₂ production and O₂ consumption within the same chamber.

Results

The system was evaluated in wheat (*Triticum monococcum*), quinoa (*Chenopodium quinoa*), and sorghum (*Sorghum bicolor*) under controlled conditions, and in *Vitis vinifera* cultivars under field conditions, in parallel with infrared gas analyzer (IRGA) measurements. CO₂ fluxes obtained with the Sensirion sensor were strongly correlated with IRGA-derived CO₂ fluxes and O₂ consumption. However, IRGA measurements generally yielded higher CO₂ fluxes, resulting in independently determined RQ values (RQ_{ind}) frequently above unity, whereas simultaneous Sensirion–PyroScience measurements yielded RQ values (RQ_{sim}) predominantly below or close to unity. Accounting for the effective respiratory leaf area substantially improved agreement between the two CO₂ measurement systems and brought RQ_{ind} estimates into closer alignment with RQ_{sim}.

Conclusions

Methodological differences in CO₂ flux estimation can propagate into substantial differences in calculated RQ. Simultaneous CO₂ and O₂ measurements within the same enclosed chamber provide a robust and accessible approach for determining leaf respiration and RQ, reducing methodological mismatches between the two respiratory fluxes and facilitating the investigation of physiological variation in respiratory stoichiometry.

Introduction

Mitochondrial respiration, or dark respiration (R_d), represents a key determinant of plant carbon balance and physiological performance [1–4]. It can be quantified either from the amount of O₂ consumed or from the amount of CO₂ released, but these two fluxes are rarely measured simultaneously. The ratio between CO₂ production and O₂ consumption, known as the respiratory quotient (RQ), provides information about the substrates and metabolic pathways supporting respiration [5–8]. While a theoretical RQ of approximately 1.0 reflects the complete, linear oxidation of carbohydrates through standard glycolysis and the tricarboxylic acid (TCA) cycle [3], empirical RQ values are often not centered on unity and frequently fall slightly below 1 [9–13]. This can reflect the predominant oxidation of substrates such as amino acids, lipids, or partially oxidized compounds, as well as processes such as incomplete decarboxylation or biosynthetic fluxes that decouple CO₂ release from O₂ consumption [9, 11, 14]. In contrast, RQ values above 1 may result from the oxidation of relatively oxidized substrates such as organic acids, or from metabolic processes that enhance decarboxylation relative to O₂ consumption [15–16] including the diversion of reducing equivalents to processes other than mitochondrial O₂ reduction, such as nitrate assimilation [17–18]. Consequently, deviations from RQ = 1 should not only be interpreted as potential indicators of shifts in substrate utilization, but also as metabolic reprogramming or changes in cellular redox balance.

Currently, O₂ consumption under darkness can be assessed using highly sensitive methodologies mostly based on liquid-phase measurements, including Clark-type electrodes and optodes [19–22]. However, these approaches remain technically challenging when applied to intact tissues or under field conditions [21, 23–25]. In contrast, the most frequently used methodology for assessing Rd is gas-exchange analysis based on CO₂ release. Open-flow infrared gas analyzers (IRGAs) are widely adopted because they provide continuous and non-destructive measurements in intact leaves while allowing precise control of environmental conditions [24, 26–27]. However, these systems are primarily designed for photosynthesis measurements and may present important limitations when used to quantify Rd, particularly due to CO₂ diffusion from beneath the chamber gasket, especially in homobaric leaves, where the proximity of internal air spaces to the leaf surface may facilitate CO₂ diffusion beneath the gasket [28–30]. On the other hand, O₂-based approaches such as Clark-type electrodes or optodes may provide an advantageous alternative when the objective is to resolve small changes in respiratory activity or small areas that cannot be measured in the gas analyzers [20–22].

The two fluxes are therefore frequently measured sequentially or using different experimental systems [7, 31], resulting in estimates of apparent RQ that can be affected by temporal, environmental and methodological differences, potentially resulting in substantial deviations from absolute RQ [20]. Previous studies illustrate the broad variability in leaf RQ, and the potential influence of the methodology used to determine respiratory fluxes [32–33]. For instance, when CO₂ and O₂ fluxes were measured independently, Watanabe et al. [34] reported RQ values of approximately 1.2–1.4. Similarly, Fan et al. [20] measured CO₂-based respiration using an IRGA and O₂ consumption independently using a gas-phase fluorophore sensor, obtaining apparent RQ values predominantly above unity and reaching up to 2.6. Simultaneous measurements of both gases have also revealed substantial biological variation in RQ. Averill and ap Rees [35] obtained RQ values of approximately 0.8–1.1 from simultaneous measurements CO₂ production and O₂ consumption by Warburg manometry in excised leaves, whereas Trethewey and ap Rees [36] reported RQ values of approximately 0.5–1.4 using the same approach. In contrast, Kruse et al. [37], using differential scanning calorimetry to quantify both respiratory CO₂ release and O₂ consumption, reported RQ values ranging from approximately 1.0 to 2.5. More sophisticated approaches have been developed to simultaneously quantify small changes in O₂ and CO₂ exchange, including highly sensitive differential gas-analysis systems [38–39], subsequently applied to intact plants [39], and membrane-inlet mass spectrometry [40–41]. In addition, cavity-enhanced Raman multi-gas spectrometry (CERS) enables continuous and non-consumptive monitoring of O₂ and CO₂ within the same chamber and has been applied to determine respiratory rates and RQ dynamics in plants [42–43]. These approaches demonstrate the advantages of determining both respiratory fluxes simultaneously from the same biological material, but generally require specialized and relatively costly instrumentation, limiting their routine application and portability. Collectively, these studies highlight the need for versatile, accessible and portable systems capable of simultaneously measuring O₂ and CO₂ exchange, which would facilitate the generation of robust RQ datasets [8].

CO₂ measurements in plant gas-exchange studies predominantly rely on relatively expensive IRGAs, whereas low-cost gas-phase O₂ sensors based on oxygen-sensitive fluorophores are becoming increasingly available. This disparity highlights the potential of combining affordable CO₂ and O₂ sensors to develop compact, accessible and portable gas-phase systems for simultaneous RQ measurements, particularly when the primary focus is on respiration rather than photosynthesis [8, 44]. In the present study, we developed a compact gas-phase system that enables that combines commercially available O₂ (Pyroscience) and CO₂ (Sensirion) sensors to simultaneously measure O₂ consumption and CO₂ production within the same closed chamber (Fig. 1). Using this system, we conducted three experiments encompassing C3 and C4 species and contrasting environmental conditions (diel cycles, drought and temperature). A particular focus of this study was to evaluate the performance of the low-cost CO₂ sensor by comparing its measurements with those obtained using conventional IRGA systems. Under controlled growth-chamber conditions, experiments were conducted in crop and pseudocereal species, including wheat (*Triticum monococcum*), sorghum

(*Sorghum bicolor*), and quinoa (*Chenopodium quinoa*). Under field conditions, experiments were conducted in different genotypes of *Vitis vinifera*. Based on previous studies highlighting the methodological advantages of determining CO₂ production and O₂ consumption simultaneously from the same biological material [39, 41–43], we hypothesized that this compact sensor-based system would provide robust simultaneous RQ estimates capable of resolving biologically meaningful variation among species and environmental conditions. We further hypothesized that differences between these simultaneous RQ estimates and RQ calculated from independently measured CO₂ and O₂ fluxes could be partly explained by an overestimation of CO₂ production rates obtained using IRGA when CO₂ diffusion from beneath the chamber gasket is ignored.

Material and Methods

Plant material, growth conditions and experimental design

For experiment 1, at the university of Granada (UGR), plants of quinoa (*Chenopodium quinoa*), sorghum (*Sorghum bicolor*), and wheat (*Triticum monococcum*) were grown in pots with a volume of 0.5–1.0 L, containing a 3:1 (v/v) mixture of peat and perlite. Plants were maintained in a controlled-environment growth chamber under a 16 h light/8 h dark photoperiod at 25/20°C (day/night), with a photosynthetic photon flux density (PPFD) of 700 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during the light period. The experimental design consisted of ten pots per species, containing three plants per pot for Quinoa and Sorghum ($n = 30$ per species), and seven plants per pot for wheat, the latter to ensure sufficient biomass for measurements. Plants were grown for four weeks and received one irrigation with 50% Hoagland nutrient solution during the growth period, followed by irrigation every 2–3 days with distilled water to field capacity. Once plants reached the vegetative phase at 30 days, fully developed leaves from 4–6 individual plants per species were selected for simultaneous Rd measurements of O₂ consumption (Pyroscience) and CO₂ production (Sensirion/IRGA). The midday measurements were taken between 9:00–13:00 h. Nighttime measurements were started at 20:00–00:00 h. All measurements were performed within a temperature range of 23–25°C.

In experiment 2, to assess the effect of water stress and genotype on leaf respiration, *Vitis vinifera* cultivars Manto negro and Callet were studied during July and August 2026 in a commercial vineyard located in Santa Eugenia (Mallorca, Spain). The vines were grown in a clay-loamy soil with a maximum depth of approximately 1.5 m and were grafted onto 110-Richter rootstock. Plants were spaced 1 m apart within rows, with 2.5 m between rows, and were trained to bilateral cordons and spur pruned, with 12 shoots per vine. Cultural practices, including irrigation, pruning, defoliation, and pest and disease management, were applied uniformly to both cultivars. Plants from 2–3 rows per cultivar were selected for leaf sampling throughout the experiment. Irrigation was scheduled based on crop evapotranspiration, calculated using a crop coefficient and evapotranspiration data recorded by a nearby meteorological station. Two water treatments were established for each cultivar: well-watered (WW) and water-stressed (WS) for two weeks, following the approach described by Tortosa et al. [45]. Midday leaf water potential was measured using a pressure chamber (Model1000; PMS Instrument Company, Albany, NY, USA) as previously described [46], and in WS plants this parameter reached approximately -1.4 MPa. Leaves from 4–6 plants of each species were selected for respiratory measurements using the simultaneous PyroScience O₂/Sensirion CO₂ system and, independently, the IRGA-based approach described below. Measurements were taken between 9:00–14:00 h. Sensirion/ Pyroscience measurements were performed in a shaded location at temperatures ranging from 27 to 29°C.

In August 2026, the third experiment was conducted with *Vitis vinifera* cv. Garnacha at the experimental field of the University of the Balearic Islands (UIB), Mallorca, Spain. The vineyard was established in 2009, with vines grafted onto 110-Richter rootstock, planted at 1 m spacing within rows and 2.5 m between rows, and trained to bilateral cordons and spur pruned. Plants were subjected to water stress following the same water-withholding and monitoring procedure described for Experiment 2. At the time of measurements, water-stressed plants had reached midday leaf water

potential values of approximately -1.4 MPa, comparable to those observed in the water-stressed vines of Experiment 2. To assess the short-term effect of measurement temperature on leaf respiration, simultaneous measurements of O_2 consumption (PyroScience) and CO_2 production (Sensirion/IRGA) were first performed under field conditions on leaves from water-stressed plants, at ambient temperatures ranging from 30 to 32°C (approximately 31°C). Leaves from the same water-stressed plants were subsequently sampled and immediately transferred to a temperature-controlled laboratory maintained at 21°C , where the same simultaneous measurements of O_2 consumption and CO_2 production were performed. Although leaves were detached for laboratory measurements, preliminary tests showed comparable respiration rates between intact and detached leaves, suggesting that leaf excision had a negligible effect on respiratory activity under the conditions tested. During measurements, detached leaves were kept hydrated by immersing their petioles in a container of water. Measurements were taken between $9:00$ – $13:00$ h.

Simultaneous O_2 and CO_2 exchange measurements using a gas-phase enclosed cuvette system

Our gas phase system was designed to enable simultaneous, high-resolution measurements of O_2 consumption and CO_2 production in leaves within the same closed chamber (Fig. 1). The system is based on an airtight stainless-steel respiration cuvette, similar to those previously used in isotope ratio mass spectrometry measurements [47]. The cuvette has an external diameter of 8 cm and a height of 4 cm, with an internal spherical cavity measuring 4 cm in diameter and 2 mm in depth. The total internal volume of the system (2.95 mL), including the spherical cavity and the interconnected spaces leading to the gas sensors, was determined gravimetrically by completely filling these spaces with distilled water (assumed density of 1.0 g mL $^{-1}$) and measuring the mass of water required to fill them. The cuvette is equipped with an optical oxygen sensor (OXROB10; PyroScience) and a miniaturized CO_2 sensor (SCD41; Sensirion), both interfaced with a microcontroller-based data acquisition system (Arduino and PyroScience platforms). The CO_2 sensor was operated using official Sensirion libraries in the Arduino environment, with Automatic Self-Calibration (ASC) enabled to maintain measurement accuracy and baseline stability through periodic adjustment of the reference baseline based on exposure to ambient CO_2 concentrations (~ 400 – 420 ppm). The optical oxygen sensor was calibrated using ambient air as a reference, corresponding to an atmospheric oxygen concentration of approximately 20.95% . In addition, the sensor modules include integrated temperature (Pt100; PyroScience) and atmospheric pressure sensors (PyroScience). For measurements performed in plants grown under chamber and field conditions, a leaf sample consisting of a disk of 11.3 cm ^{2} and 9.6 cm ^{2} , respectively, was introduced into the cuvette (Fig. 1). Oxygen and carbon dioxide concentrations were continuously recorded over approximately 10 minutes; the first 3 min were discarded as an equilibration period. This measurement window also allowed sufficient CO_2 accumulation and O_2 depletion while preserving the linearity of gas exchange rates, which was visually assessed by confirming that a linear regression reference line closely overlapped with the measured gas concentration data. Oxygen concentration was recorded at 1 s intervals, whereas CO_2 concentration was measured every 4 s. Leaves were dark-adapted for 15 – 20 min before measurements. The cuvettes were sealed using silicone sealant and fitted with O-rings to ensure airtightness and prevent gas leakage. Prior to measurements, the cuvettes were equilibrated to ambient temperature to minimize thermal drift, and blank measurements (empty cuvettes) were performed periodically to verify the integrity of the seals and detect potential gas leakage. This system was designed with portability in mind, as all sensors are powered through a portable computing unit.

The optode quantifies changes in gas-phase O_2 concentration from the O_2 -dependent decay of the fluorescence signal. The oxygen consumption rates (RO_2 , $\mu\text{mol m}^{-2}\text{s}^{-1}$) were calculated using ideal gas law, following previous studies [32–44]:

$$R = (P \cdot V \cdot SO / R \cdot T \cdot A) \cdot 10 \quad (1)$$

In this equation, P represents the total ambient partial pressure, which is 101.325 kilopascal (kPa). V denotes the total internal gas-space volume of the respiratory cuvette (mL), measured without leaf tissue. The volume occupied by the leaf tissue was not quantified or subtracted. SO_2 denotes the slope of O_2 decay over time (e.g., $O_2 = \text{ppm } O_2 \text{ s}^{-1}$), as determined by the optode sensor as percentage of O_2 in the gas phase (% O_2). The slope was divided by 100 to convert O_2 percentage into mole fraction before calculating the molar flux. R stands for the gas constant, which is $8314 \text{ cm}^3 \text{ kPa K}^{-1} \text{ mol}^{-1}$. T represents the temperature in Kelvin (K), and A is the total projected area of leaf discs (m^2). The factor 10^{-6} converts concentration from ppm to mole fraction. Similarly, as CO_2 evolved by the tissue and accumulates within the sealed cuvette, a positive slope ($SCO_2, \text{ppm } CO_2 \text{ s}^{-1}$) was used to calculate the CO_2 evolution rate ($RCO_2, \mu\text{mol m}^{-2} \text{ s}^{-1}$) was calculated as:

$$R = (P \cdot V \cdot SCO / R \cdot T \cdot A) \cdot 10 \quad (2)$$

Both temperature and atmospheric pressure recorded by the integrated sensors during each measurement were used for conversion of gas concentration changes to molar fluxes. Leaf area was either determined using ImageJ software or directly defined by sampling leaf tissue using a punch to excise circular leaf discs of known area. Depending on the experimental setup, leaf discs with predefined areas of 11.3 or 9.62 cm^2 were used for respiration measurements.

Finally, the simultaneously respiratory quotient (RQ_{sim}) was calculated as the simple ratio of these two rates:

$$RQ_{\text{sim}} = R_{CO_2} / R_{O_2} \quad (3)$$

CO₂ exchange measurements using an Open-Flow Infrared Gas Analyzer System

Dark respiration ($R_{CO_2\text{IRGA}}$) was determined in the youngest fully developed leaves of at least four plants of *C. quinoa*, *S. bicolor*, *T. monococcum*, and *V. vinifera* using a LI-6400 gas-exchange system (LI-COR Inc., Lincoln, NE, USA) equipped with a 2 cm^2 leaf chamber. These measurements were performed in parallel with the O_2 consumption and CO_2 production determinations obtained using the PyroScience and Sensirion systems, respectively. All measured leaves were larger than the LI-6400 chamber area. Across experiments, LI-6400 operating conditions were adjusted to the specific environmental and experimental conditions under which respiration was assessed. Reference CO_2 concentration was maintained between 400 and 420 $\mu\text{mol } CO_2 \text{ mol}^{-1} \text{ air}$, while air flow rates ranged from 150 to 300 $\mu\text{mol air s}^{-1}$. Block temperature was set to match, as closely as possible, the corresponding measurement conditions of each experiment. Specifically, measurements were performed at 25°C in Experiment 1 and at 27°C in Experiment 2, whereas in Experiment 3 measurements were conducted at 31°C under field conditions and at 21°C following transfer to the temperature-controlled laboratory. Before respiration measurements, leaves were dark-adapted for 15–20 min, and gas-exchange rates were recorded once a steady state had been reached. Leak tests were performed daily before measurements by blowing around the chamber clamp to detect potential leaks. Additionally, blank measurements were conducted with the cuvette closed and without leaf material to verify that respiration rates remained close to zero, confirming the absence of detectable gas leakage or instrumental drift.

Finally, the independently respiratory quotient (RQ_{ind}) was calculated from CO_2 production measured with the IRGA system ($R_{CO_2\text{IRGA}}$) and O_2 consumption measured with the PyroScience system (R_{O_2}). Because IRGA and PyroScience measurements were not performed on the same individual leaves, individual CO_2 and O_2 measurements could not be

directly paired. Instead, measurements with the different systems were performed on leaves sampled from nearby positions within the same plants. Therefore, for each treatment, R_{O_2} values obtained with PyroScience were averaged, and this treatment-level mean O_2 consumption rate was subsequently used to calculate the AP_{RQ} for each individual R_{CO_2LICOR} measurement:

$$RQ_{ind} = R_{CO_2LICOR} / R_{O_2} \quad (4)$$

Statistical analyses

All statistical analyses and data visualization were performed using R software (v. 4.3.2; R Core [48]). Statistical analyses were conducted independently for each experiment. Differences among experimental treatments in respiratory variables, including CO_2 production, O_2 consumption, and respiratory quotient (RQ), were assessed using one-way analysis of variance (ANOVA). When significant treatment effects were detected, pairwise comparisons were performed using estimated marginal means with adjustment for multiple comparisons, and homogeneous groups were represented using compact letter displays (CLDs). Statistical significance was accepted at $p < 0.05$. Differences between alternative methodological approaches for estimating respiratory CO_2 fluxes and RQ (i.e., IRGA vs. Sensirion and IRGA/PyroScience vs. Sensirion/PyroScience, respectively) were evaluated using Student's t-tests. Data distributions were visualized using boxplots, together with treatment means and their associated standard errors (SE). Relationships among respiratory measurements obtained with the different systems were assessed using ordinary least-squares linear regression. Regressions were fitted using treatment mean values, and the uncertainty associated with each mean was represented by horizontal and vertical error bars corresponding to the SE of the variables plotted on each axis. Regression equations, coefficients of determination (R^2), and associated p-values were reported to describe the strength and significance of these relationships. Data processing and visualization were performed using the ggplot2, dplyr, tidyr, and patchwork packages, whereas estimated marginal means, multiple comparisons, and compact letter displays were obtained using the emmeans and multcomp packages.

Results

Respiration measurements under controlled growth-chamber conditions

O_2 consumption measured with the PyroScience sensor ranged from 0.68 to 1.70 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$ (Table 1). *Quinoa* exhibited the highest O_2 consumption, with 1.41 ± 0.07 and $1.70 \pm 0.19 \mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$ at midday and nighttime, respectively. In contrast, *Sorghum* showed the lowest values, 0.68 ± 0.04 and $0.70 \pm 0.05 \mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$, while wheat showed intermediate consumption of 0.81 ± 0.09 and $0.84 \pm 0.04 \mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$. In previous studies, measurements performed in different wheat species and environments showed a considerably broader range of 0.25–4.0 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$ by using hyperspectral reflectance or fluorophore sensitive to O_2 quenching [49–51]. For *Sorghum*, using an O_2 -sensitive fluorophore, Fan et al. [20] reported O_2 -based dark respiration rates of approximately 1.0–2.0 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$. In comparison, the values in our experiment (approximately 0.70 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$) are somewhat lower, but still within the broader range of O_2 -based respiration reported for C_4 grasses (0.5–1.0 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ s}^{-1}$) in the same study (Table 1A).

Respiratory CO_2 release differed among species and, to a lesser extent, between measurement times (MD and NT) under controlled growth-chamber conditions (Table 1). Across the three species, IRGA measurements ranged from 0.99 to 1.57 $\mu\text{mol } CO_2 \text{ m}^{-2} \text{ s}^{-1}$, whereas Sensirion yielded consistently lower values, ranging from 0.52 to 1.07 $\mu\text{mol } CO_2 \text{ m}^{-2} \text{ s}^{-1}$. Nevertheless, both systems captured a similar overall pattern among species, with quinoa showing the highest CO_2 release and wheat and sorghum generally displaying lower rates. In quinoa, IRGA measurements were 1.57 ± 0.18 and $1.46 \pm 0.10 \mu\text{mol } CO_2 \text{ m}^{-2} \text{ s}^{-1}$ at MD and NT, respectively, compared with 0.96 ± 0.08 and $1.07 \pm 0.15 \mu\text{mol } CO_2 \text{ m}^{-2} \text{ s}^{-1}$

obtained with Sensirion, corresponding to differences of approximately 27–39% between methods. A larger methodological divergence was observed in wheat, particularly at MD, where IRGA recorded $1.37 \pm 0.11 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ compared with $0.52 \pm 0.05 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ with Sensirion; at NT, the corresponding values were 1.06 ± 0.06 and $0.61 \pm 0.04 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Similarly, sorghum showed IRGA rates of 0.99 ± 0.05 and $1.07 \pm 0.10 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at MD and NT, respectively, whereas Sensirion yielded approximately half these values (0.55 ± 0.03 and $0.58 \pm 0.05 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) (Table 1A). Thus, although both approaches captured broadly consistent interspecific patterns in respiratory activity, the magnitude of the difference between IRGA- and Sensirion-derived CO_2 fluxes varied among species.

The differences between CO_2 fluxes resulted in a clear separation between both RQ methodologies (Table 1; Fig. 1A,B). RQ_{ind} , calculated from IRGA CO_2 release and PyroScience O_2 consumption, ranged from 0.86 to 1.69 and was consistently higher than the corresponding RQ_{sim} in the treatments showing a significant difference. The highest RQ_{ind} was observed in wheat at MD (1.69 ± 0.14), followed by sorghum at NT (1.52 ± 0.14) and sorghum at MD (1.45 ± 0.08). In contrast, RQ_{sim} calculated from simultaneous Sensirion–PyroScience measurements ranged from 0.62 to 0.82. Quinoa showed the lowest absolute RQ at nighttime (0.62 ± 0.02), whereas sorghum showed the highest values, 0.81 ± 0.01 and 0.82 ± 0.02 at MD and NT, respectively. No statistically significant differences in CO_2 evolution rates were detected between the two diurnal time points for any of the studied species, regardless of whether measurements were performed using the IRGA or the Sensirion sensor. Similarly, no significant differences were observed in both midday and nighttime values for either RQ_{ind} or RQ_{sim} . The sole exception was a decrease in RQ_{ind} during nighttime in quinoa. This RQ_{ind} decline is probably caused by gasket artifacts in the IRGA chamber, rather than a shift in the substrate oxidized.

Respiration measurements in *Vitis vinifera* under field conditions

Because direct measurements of leaf O_2 consumption (area basis) in *V. vinifera* are limited, values from closely related grapevine cultivars were evaluated for context. Field measurements revealed that PyroScience O_2 consumption ranged from 0.71 to $2.48 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ across cultivars and treatments (Table 2). Callet recorded the highest O_2 demand, with 2.48 ± 0.12 and $2.37 \pm 0.10 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ under well-watered (WW) and water-stress (WS) conditions, respectively. In contrast, Manto Negro exhibited lower O_2 uptake under WW conditions ($1.30 \pm 0.11 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$), which increased significantly to $1.62 \pm 0.17 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ under WS. Grenache demonstrated sharp temperature sensitivity, elevating its O_2 consumption from $0.71 \pm 0.05 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ at 21°C to $1.64 \pm 0.12 \mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ at 31°C (Table 1B).

Field-based respiratory CO_2 release in *V. vinifera* exhibited substantially greater variability than observed in growth-chamber experiments (Table 2). Across cultivars, IRGA CO_2 fluxes spanned from 1.07 to $3.54 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, whereas Sensirion measurements registered a lower overall range of 0.48 to $2.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Callet yielded the highest CO_2 release with IRGA (3.54 ± 0.12 under WW; $3.17 \pm 0.26 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under WS) and relatively high, stable values with Sensirion (2.02 ± 0.19 under WW; $2.00 \pm 0.22 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under WS). In Manto Negro, water stress induced a significant rise in IRGA rates (from 1.48 ± 0.07 under WW to $2.71 \pm 0.27 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under WS), while Sensirion recorded 1.20 ± 0.09 and $1.32 \pm 0.15 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under WW and WS, respectively. Grenache showed significant thermal drive in both sensors, increasing from 1.07 ± 0.06 (21°C) to $2.08 \pm 0.11 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at 31°C (IRGA) and from 0.48 ± 0.02 (21°C) to $1.49 \pm 0.15 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ at 31°C (Sensirion) (Table 1B).

Similar to experiment 1, these contrasting CO_2 estimates generated a distinct divergence between apparent (IRGA CO_2 / PyroScience O_2) and absolute (Sensirion CO_2 / PyroScience O_2) respiratory quotients (Table 2; Fig. 1A,B). Apparent RQ spanned from 1.14 to 1.67 across treatments, whereas absolute RQ showed values below unity (0.68 to 0.95). In Callet, apparent RQ reached 1.43 ± 0.05 (WW) and 1.34 ± 0.11 (WS), compared to absolute RQ values of 0.81 ± 0.05

and 0.84 ± 0.07 , respectively. Manto Negro exhibited a significant increase in apparent RQ from 1.14 ± 0.05 (WW) to 1.67 ± 0.17 (WS), while its absolute RQ remained around 0.8–1.0 (0.95 ± 0.11 under WW; 0.81 ± 0.01 under WS). Grenache exhibited the most pronounced temperature-dependent shift: at 21 °C, apparent RQ was 1.51 ± 0.08 versus an absolute RQ of 0.68 ± 0.03 , whereas at 31 °C, the values converged closer to 1.37 ± 0.07 (apparent) and 0.93 ± 0.11 (absolute). A lack of statistically significant differences between the two CO₂ and RQ metrics were confirmed for most treatments, with exceptions noted in Manto Negro under WW conditions and Grenache at 31 °C. The disparity between RQ estimates was not uniform across treatments, scaling directly with the magnitude of disagreement between the underlying CO₂ sensors. The most extreme divergence occurred in treatments where IRGA CO₂ release far exceeded the simultaneous Sensirion output, most notably in Manto Negro under WS conditions and Grenache at 21 °C. Conversely, when IRGA and Sensirion CO₂ estimates aligned more closely, the resulting apparent and absolute RQ values converged accordingly.

Relationships among respiratory CO₂ and O₂ fluxes across measurement systems

To evaluate the degree of agreement between the different measurement systems, linear regressions were performed using the mean values for each treatment across all experiments. The comparison between respiratory CO₂ fluxes measured with IRGA and the Sensirion sensor demonstrated a positive and highly significant relationship ($R^2=0.88$; Fig. 3C). Both systems captured consistent relative variations in respiratory activity across treatments. However, the slope of 0.59—substantially below the theoretical 1:1 unity line— highlights a proportional overestimation of CO₂ release by the IRGA maybe driven by the multicomponent leaks artifacts [24, 28] (Fig. 3C, Supplementary Fig. 1). A similar pattern emerged when comparing CO₂ production against O₂ consumption measured with PyroScience (Fig. 3A). When pairing independent IRGA CO₂ fluxes with PyroScience O₂ uptake, the relationship was highly significant ($R^2=0.85$). The slope exceeding unity (1.28) reveals higher rates of CO₂ release measured with the IRGA than rates of O₂ consumption across the respiratory spectrum, introducing a systematic excess of CO₂ relative to O₂ (Fig. 3A). On the other hand, simultaneous measurements conducted within the same chamber using Sensirion and PyroScience yielded a stronger linear coupling ($R^2=0.94$; Fig. 3B). The higher coefficient of determination and a slope closer to unity (0.85) demonstrate that CO₂ and O₂ exchanges measured simultaneously in an integrated volume produces less error than when combining independent gas exchange systems (Fig. 3A,B). This pattern is reinforced when comparing the CO₂ values of both instruments, in which prior a gasket leak correction the slope of both IRGA and Sensirion CO₂ was 0.56, and after the 50% of area correction, this slope change to unity (1.01), indicating that changes observed in the CO₂ values are not due a biological difference, confirming that correcting for diffusion leakage is essential for obtaining realistic CO₂ values, and miniaturized sensor is capable of capture dynamic changes in CO₂ respiration with high accuracy.

Discussion

Comparison of respiratory CO₂ fluxes and the influence of effective leaf area

A central result of this study is that the CO₂ fluxes obtained with the IRGA and the miniaturized Sensirion sensor were strongly correlated across the different species and experimental conditions, but that the two approaches did not initially provide equivalent estimates of respiratory CO₂ release (Fig. 2–4). IRGA measurements were systematically higher than those obtained with the Sensirion sensor, resulting in a significant difference between methods when the complete dataset was compared (Figs. 3 and 5). Despite this difference in absolute magnitude, the two methods captured a common pattern of variation among treatments, as indicated by the significant linear relationship between them (Fig. 3C). However, the slope of the relationship between Sensirion- and IRGA-derived CO₂ fluxes was

substantially below unity (0.59), indicating that the discrepancy between methods increased with respiratory rate. Thus, although both systems captured similar relative variation in respiratory activity across treatments, their estimates increasingly diverged at higher CO₂ fluxes. One potential explanation for this proportional divergence is an overestimation of respiratory CO₂ release by the open-flow clamp-on IRGA system due to CO₂ produced by leaf tissue beneath the chamber gasket and diffusing into the chamber [28–30] (Figs. 2, 4 and 5). Importantly, despite their systematically higher values relative to the Sensirion measurements, IRGA-derived CO₂ fluxes remained within previously reported physiological ranges for the species examined. In *Chenopodium quinoa*, MD and NT rates (1.57 ± 0.18 and 1.46 ± 0.10 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) closely match literature values for Rd (~ 1.19 – 1.85 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; [52] (Table 1A). Similarly, field values in *Vitis vinifera* (1.07 to 3.54 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) align with published ranges under contrasting water and temperature regimes (~ 0.5 to 3.0 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; [53–54]. Likewise, rates measured in *Sorghum bicolor* and *Triticum monococcum* were consistent with previously reported values [20, 51] (Table 1B). Thus, the discrepancy between methods cannot be identified simply by comparing IRGA-derived rates with the range of respiratory values previously reported in the literature.

To evaluate if some IRGA chamber artifacts causes the disparity between methods, we apply a respiratory-area correction to the IRGA data (Figs. 2 and 4). The correction is based in the assumption that the leaf tissue covered by the gasket continues to respire and that approximately 50% of the CO₂ produced by this tissue diffuses towards the chamber, while the remaining 50% diffuses towards the outside [28]. Accordingly, one-half of the gasket-covered area was incorporated into the effective respiratory area used to normalize the IRGA flux (Additional Fig. 1). This represents a first-order approximation based on a fixed contribution of gasket-covered tissue rather than a direct measurement of lateral CO₂ diffusion, which is expected to vary among leaves and species. Nevertheless, applying this correction reduced IRGA-derived CO₂ fluxes by approximately 42% (Additional Fig. 1) and markedly improved agreement with the Sensirion measurements. The relationship between corrected IRGA and Sensirion fluxes remained highly significant ($R^2 = 0.86$, $p < 0.001$, Fig. 5B), with the regression slope shifted from 0.59 (Fig. 3C) to 1.01 after correction. Close agreement was observed across several species and treatments, although residual differences remained under some conditions, reaching approximately up to 0.34 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$. Thus, the 50% assumption should not be interpreted as a universal correction factor, as lateral CO₂ diffusion beneath the gasket is a physical mass-transport artifact governed by anatomical and diffusional properties, including mesophyll resistance, leaf porosity and vascular anatomy, which can vary considerably among species [28]. Rather, the correction illustrates that respiration by gasket-covered tissue can account for a substantial part of the systematic discrepancy between the two measurement approaches. The convergence of the regression slope towards unity, together with improved agreement between IRGA- and Sensirion-derived RQ estimates after correction (Fig. 3C and 5) provides strong evidence that gasket-associated lateral CO₂ diffusion contributes to the original discrepancy. In contrast, enclosing an entire leaf disc within the hermetic Sensirion/PyroScience chamber avoids this particular source of error.

Physiological variation in respiratory quotient and implications for respiratory metabolism

Because RQ is calculated as the ratio between CO₂ production and O₂ consumption, relatively small differences in the estimated CO₂ flux can be amplified in the resulting RQ, particularly when respiration rates are low. Thus, differences of only a few tenths of $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ arising from the calculation of the effective IRGA respiratory area can produce a much larger relative change when expressed as a CO₂/O₂ ratio (Table 1–2; Figs. 2 and 4). This was evident when the conventional IRGA area was used: the resulting independent RQ estimates (RQ_{ind}) were predominantly above unity, with several treatments exceeding 1.6, similar to the range of 1.1 to 2.6 as previously reported [20]. Although RQ values above unity are physiologically possible, such high values would imply a substantial contribution of highly oxidized respiratory substrates, particularly organic acids [8], and contrast with the RQ values around or slightly below unity

commonly reported for leaves [9–13]. Moreover, plant respiration can be supported by a diverse range of substrates, including carbohydrates, organic acids, amino acids and fatty acids, whose relative contribution varies with metabolic and environmental conditions [55]. In particular, amino acids can contribute to mitochondrial respiration both under normal conditions and when carbohydrate availability becomes limiting, further illustrating that respiratory metabolism cannot be inferred from carbohydrate oxidation alone [55]. In contrast, RQ values obtained from simultaneous Sensirion–PyroScience measurements (RQ_{sim}) were consistently lower and generally centered below or close to unity, in agreement with previous simultaneous measurements of CO_2 production and O_2 consumption in leaves [12, 39–41, 43, 56]. The magnitude of the discrepancy between RQ_{ind} and RQ_{sim} varied considerably among species and treatments, being particularly pronounced in wheat, sorghum, water-stressed Manto Negro and Grenache at 21°C, while it was smaller in quinoa and in treatments where the two CO_2 estimates became more similar after the respiratory-area correction. The marked change observed in Grenache, from an RQ_{sim} of 0.93 at 31°C to 0.68 when measured immediately at 21°C, is more likely to reflect the immediate temperature dependence of respiratory CO_2 production and O_2 consumption than a major reorganization of respiratory substrate availability, because leaves acclimated to 31°C were transferred directly to 21°C for measurement without allowing thermal acclimation (Table 2). When compared with theoretical or experimentally reported RQ values for different respiratory substrates, several RQ_{ind} values fell outside the values compiled in the literature (“nr” in Table 2; [8], whereas all RQ_{sim} values could be linked to previously reported substrates (Table 2; [8]. Overall, RQ_{sim} values fell within reported ranges. Overall, the RQ_{sim} values were compatible with contributions from amino acids, carbohydrates, and/or lipids, whereas the higher RQ_{ind} values would imply a greater contribution from organic-acid metabolism (Table 2A,B). These associations should not, however, be interpreted as direct identification of the substrates being respired, because multiple substrates and metabolic pathways contribute simultaneously to respiratory CO_2 release and O_2 consumption, and their use varies with physiological and environmental conditions [55]. In well-watered Manto Negro, for instance, an RQ_{sim} close to unity (0.95) is consistent with a substantial contribution of carbohydrate oxidation. By contrast, the lower RQ_{sim} values observed in most other treatments indicate greater O_2 consumption relative to CO_2 release and are therefore consistent with contributions from other respiratory substrates and/or metabolic processes in addition to carbohydrate oxidation. Importantly, simultaneous measurement of CO_2 and O_2 exchange minimizes temporal, biological-sample and instrumental mismatches between the two fluxes, but does not necessarily make the resulting RQ_{sim} equivalent to the biochemical RQ of mitochondrial substrate oxidation. Whole-leaf CO_2 release and O_2 uptake can also be influenced by metabolic processes other than mitochondrial substrate oxidation, including CO_2 fixation by carboxylating reactions, diversion of reducing equivalents to processes such as nitrate assimilation, and non-mitochondrial O_2 consumption [7, 17–18]. Thus, RQ_{sim} should be interpreted as a robust measure of whole-tissue CO_2/O_2 exchange stoichiometry rather than as an unequivocal indicator of the substrates oxidized by mitochondrial respiration.

The difference between these two approaches (RQ_{ind} vs RQ_{sim}) was highly significant, illustrating how methodological differences in CO_2 estimation can translate into apparently large differences in RQ (Figs. 2 and 4). After applying the area correction, the RQ values calculated from IRGA CO_2 production and PyroScience O_2 consumption (RQ_{ind}) were markedly closer to those obtained from the simultaneous Sensirion–PyroScience measurements (RQ_{sim} , Fig. 3A,B, and 5). More importantly, most of the corrected values were below unity, with only wheat measured during the daytime exceeding 1. This corrected range is also consistent with RQ values reported in studies using approaches that allow simultaneously or directly coupled measurements of respiratory CO_2 production and O_2 consumption, including cavity-enhanced Raman multigas spectrometry (CERS) [12, 39, 41, 43, 56]. The relationships between CO_2 and O_2 measurements provide further support for this interpretation (Fig. 4A,B). Although both IRGA and Sensirion CO_2 fluxes were strongly related to O_2 consumption measured with PyroScience, the Sensirion–PyroScience relationship showed a stronger fit than the corresponding IRGA–PyroScience relationship (Fig. 3A,B). Moreover, the Sensirion–PyroScience regression had a slope closer to the theoretical 1:1 relationship than that observed for IRGA versus PyroScience after

respiratory-area correction (Fig. 3C, and 5A). These differences indicate that the CO₂ production measured simultaneously with the Sensirion sensor was more tightly coupled to O₂ consumption than the independently obtained IRGA flux, even after applying a respiratory-area correction to account for differences in the leaf area used for flux calculations.

Conclusions

Combining simultaneous CO₂ and O₂ gas-exchange measurements within an enclosed cuvette provides a robust and complementary approach for characterizing Rd, and changes in respiratory stoichiometry across species and environmental conditions. Simultaneous measurements of CO₂ production and O₂ consumption within the same cuvette yielded RQ_{sim} values that were lower than the RQ_{ind} values calculated from independent IRGA and optode measurements. This divergence could largely be explained by an overestimation of CO₂ production rates in clamp-on IRGA systems when lateral gas diffusion and respiration from tissue beneath the chamber gasket are not considered. Mathematically incorporating this gasket-derived contribution substantially improved agreement between CO₂ fluxes measured with both systems and brought RQ_{ind} estimates into closer alignment with RQ_{sim} measurements. These results may have implications beyond the estimation of Rd itself. Because Rd is commonly used as an input parameter in the estimation of other gas-exchange traits, including carbon balance, mesophyll conductance and maximum carboxylation rate, systematic errors in Rd associated with the effective respiratory area could propagate into their estimation. Beyond these methodological implications, the accessibility of simultaneous RQ measurements opens opportunities to investigate the metabolic basis of variation in respiratory stoichiometry. Combining RQ_{sim} with metabolomic profiling could help relate changes in CO₂/O₂ stoichiometry to changes in respiratory substrates and primary metabolic pathways, while also enabling new questions about how respiratory metabolism is reorganized across species, developmental stages and environmental conditions. Complementary approaches such as oxygen-isotope fractionation (Δ¹⁸O), which enables partitioning of mitochondrial O₂ consumption between the cytochrome and alternative oxidase pathways, could further help disentangle variation in whole-tissue RQ from changes in mitochondrial respiratory metabolism and from other processes contributing to net CO₂ and O₂ exchange.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

N.F.-D.S. acknowledges support through the Ramón y Cajal contract RYC2023-044592-I, funded by the Agencia Estatal de Investigación (AEI; 10.13039/501100011033). Funding for open access charge: Universidad de Granada/CBUA.

Author Contribution

J.O. contributed to experimental design, data collection, data analysis, and manuscript writing. I.T. performed experimental measurements, contributed to data collection and analysis, and participated in manuscript writing. I.F.-S., J.F., and M.R.-C. contributed to the critical revision, editing, and improvement of the manuscript. T.I.F. contributed to the technical design and development of the experimental chambers and measurement system. N.F.-D.S. conceived and designed the study, contributed to the technical development and implementation of the measurement system, secured funding for the acquisition of equipment and materials, and led manuscript writing and revision.

Acknowledgement

We are very grateful to Biel Martorell and Mateu Fullana from the Serveis Científic-Tècnics of the Universitat de les Illes Balears for their assistance with the IRMS experiments. We thank Rafael Montero Silvestre from Bodega Son Juliana for his technical assistance and vineyard management. We also thank Mr. Miquel Truyols and the staff of the UIB Experimental Field for their support and assistance during the field experiments.

Availability of data and materials

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Atkin OK, Edwards EJ, Loveys BR. Response of root respiration to changes in temperature and its relevance to global warming. *New Phytol.* 2000;147:141–54.
2. Millar AH, Whelan J, Soole KL, Day DA. Organization and regulation of mitochondrial respiration in plants. *Annu Rev Plant Biol.* 2011;62:79–104.
3. O’Leary BM, Asao S, Millar AH, Atkin OK. Core principles which explain variation in respiration across biological scales. *New Phytol.* 2019;222:670–86.
4. Amthor JS, Bar-Even A, Hanson AD, Millar AH, Stitt M, Sweetlove LJ, Tyerman SD. Engineering strategies to boost crop productivity by cutting respiratory carbon loss. *Plant Cell.* 2019;31:297–314.
5. Platenius H. Effect of temperature on the respiration rate and the respiratory quotient of some vegetables. *Plant Physiol.* 1942;17:179–97.
6. Bower JH, Jobling JJ, Patterson BD, Ryan DJ. A method for measuring the respiration rate and respiratory quotient of detached plant tissues. *Postharvest Biol Technol.* 1998;13:263–70.
7. Hilman B, Weiner T, Haran T, Masiello CA, Gao X, Angert A. The apparent respiratory quotient of soils and tree stems and the processes that control it. *J Geophys Res Biogeosci.* 2022;127:e2021JG006676.
8. Bruhn D, Fan Y, Griffin KL, Cowan-Turner D, Scafaro AP, Møller IM, Atkin OK. Importance of the leaf respiratory quotient. *Physiol Plant.* 2025;177:e70235.
9. Lambers H. The functional equilibrium, nibbling on the edges of a paradigm. *Neth J Agric Sci.* 1983;31:305–11.
10. Seibt U, Brand WA, Heimann M, Lloyd J, Severinghaus JP, Wingate L. Observations of O₂:CO₂ exchange ratios during ecosystem gas exchange. *Glob Biogeochem Cycles.* 2004;18:GB4024.
11. Araujo WL, Tohge T, Ishizaki K, Leaver CJ, Fernie AR. Protein degradation—an alternative respiratory substrate for stressed plants. *Trends Plant Sci.* 2011;16:489–98.

12. Patterson AE, Arkebauer R, Quallo C, Heskell MA, Li X, Boelman N, Griffin KL. Temperature response of respiration and respiratory quotients of 16 co-occurring temperate tree species. *Tree Physiol.* 2018;38:1319–32.
13. Hicks Pries CE, Angert A, Castanha C, Hilman B, Torn MS. Using respiration quotients to track changing sources of soil respiration seasonally and with experimental warming. *Biogeosciences.* 2020;17:3045–55.
14. Burr GO, Miller ES. Synthesis of fats by green plants. *Bot Gaz.* 1938;99:773–85.
15. Jenkins ME, Adams MA. Respiratory quotients and Q10 of soil respiration in sub-alpine Australia reflect influences of vegetation types. *Soil Biol Biochem.* 2011;43:1266–74.
16. Berggren M, Lapierre JF, del Giorgio PA. Magnitude and regulation of bacterioplankton respiratory quotient across freshwater environmental gradients. *ISME J.* 2012;6:984–93.
17. Bloom AJ, Caldwell RM, Finazzo J, Warner RL, Weissbart J. Oxygen and carbon dioxide fluxes from barley shoots depend on nitrate assimilation. *Plant Physiol.* 1989;91:352–6.
18. Scheurwater I, Cornelissen C, Dictus F, Welschen R, Lambers H. Why do fast- and slow-growing grass species differ so little in their rate of root respiration, considering the large differences in rate of growth and ion uptake? *Plant Cell Environ.* 1998;21:995–1005.
19. Jacoby RP, Millar AH, Taylor NL. Assessment of respiration in mitochondria. In: Whelan J, Murcha MW, editors. *Plant Mitochondria: Methods and Protocols.* New York: Springer; 2015. pp. 165–85.
20. Fan Y, Tcherkez G, Scafaro AP, Taylor NL, Furbank RT, von Caemmerer S, Atkin OK. Variation in leaf dark respiration among C3 and C4 grasses is associated with use of different substrates. *Plant Physiol.* 2024;195:1475–90.
21. Sena F, Couture C, Berais-Rubio A, Millar AH, Signorelli S. Liquid-phase determination of Arabidopsis respiration and photosynthesis using Clark-type O₂ electrodes. *Plant Sci.* 2025;360:112735.
22. Fan Y, Tcherkez G, Scafaro AP, Taylor NL, Furbank RT, von Caemmerer S, Atkin OK. Diel-driven variations of leaf dark respiration and metabolite levels in C3 and C4 grasses. *Plant Physiol.* 2026;201:kiag200.
23. Hunt S. Measurements of photosynthesis and respiration in plants. *Physiol Plant.* 2003;117:314–25.
24. Flexas J, Díaz-Espejo A, Berry JA, Cifre J, Galmés J, Kaldenhoff R, Medrano H, Ribas-Carbó M. Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. *J Exp Bot.* 2007;58:1533–43.
25. Sun Y, Li M, Cheng Q, Jungbluth KH, Maack C, Buescher W, et al. Tracking oxygen and temperature dynamics in maize silage-novel application of a Clark oxygen electrode. *Biosyst Eng.* 2015;139:60–5.
26. Flexas J, Bota J, Escalona JM, Sampol B, Medrano H. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. *Funct Plant Biol.* 2002;29:461–71.
27. Douthe C, Gago J, Ribas-Carbó M, Núñez R, Pedrol N, Flexas J. Measuring photosynthesis and respiration with infrared gas analysers. In: Sánchez-Moreiras AM, Reigosa MJ, editors. *Advances in Plant Ecophysiology Techniques.* Cham: Springer; 2018. pp. 51–75.
28. Pons TL, Welschen RAM. Overestimation of respiration rates in commercially available clamp-on leaf chambers: complications with measurement of net photosynthesis. *Plant Cell Environ.* 2002;25:1367–72.
29. Jahnke S. Atmospheric CO₂ concentration does not directly affect leaf respiration in bean or poplar. *Plant Cell Environ.* 2001;24:1139–51.
30. Montero R, Ribas-Carbó M, Del-Saz NF, El Aou-Ouad H, Berry JA, Flexas J, Bota J. Improving respiration measurements with gas exchange analyzers. *J Plant Physiol.* 2016;207:73–7.
31. Angert A, Yakir D, Rodeghiero M, Preisler Y, Davidson EA, Weiner T. Using O₂ to study the relationships between soil CO₂ efflux and soil respiration. *Biogeosciences.* 2015;12:2089–99.

32. Scafaro AP, Negrini ACA, O'Leary B, Rashid FAA, Hayes L, Fan Y, Zhang Y, Chochois V, Badger MR, Millar AH, Atkin OK. The combination of gas-phase fluorophore technology and automation to enable high-throughput analysis of plant respiration. *Plant Methods*. 2017;13:16.
33. Bruhn D, Slot M, Mercado LM. Simple and accurate representation of cumulative midnight leaf respiratory CO₂ efflux. *Glob Change Biol*. 2024;30:e17529.
34. Watanabe CK, Sato S, Yanagisawa S, Uesono Y, Terashima I, Noguchi K. Effects of elevated CO₂ on levels of primary metabolites and transcripts of genes encoding respiratory enzymes and their diurnal patterns in *Arabidopsis thaliana*: possible relationships with respiratory rates. *Plant Cell Physiol*. 2014;55:341–57.
35. Averill RH, ap Rees T. The control of respiration in wheat (*Triticum aestivum* L.) leaves. *Planta*. 1995;196:344–9.
36. Trethewey RN, ap Rees T. A mutant of *Arabidopsis thaliana* lacking the ability to transport glucose across the chloroplast envelope. *Biochem J*. 1994;301:449–54.
37. Kruse J, Hopmans P, Adams MA. Temperature responses are a window to the physiology of dark respiration: differences between CO₂ release and O₂ reduction shed light on energy conservation. *Plant Cell Environ*. 2008;31:901–14.
38. Willms JR, Dowling AN, Dong ZM, Hunt S, Shelp BJ, Layzell DB. The simultaneous measurement of low rates of CO₂ and O₂ exchange in biological systems. *Anal Biochem*. 1997;254:272–82.
39. Cen YP, Turpin DH, Layzell DB. Whole-plant gas exchange and reductive biosynthesis in white lupin. *Plant Physiol*. 2001;126:1555–65.
40. Siebke K, Ghannoum O, Conroy JP, Badger MR, von Caemmerer S. Photosynthetic oxygen exchange in C₄ grasses: the role of oxygen as electron acceptor. *Plant Cell Environ*. 2003;26:1963–72.
41. Beckmann K, Messinger J, Badger MR, Wydrzynski T, Hillier W. On-line mass spectrometry: membrane inlet sampling. *Photosynth Res*. 2009;102:511–22.
42. Keiner R, Frosch T, Massad T, Trumbore S, Popp J. Enhanced Raman multigas sensing—a novel tool for control and analysis of ¹³CO₂ labeling experiments in environmental research. *Analyst*. 2014;139(16):3879–84.
43. Hanf S, Fischer S, Hartmann H, Keiner R, Trumbore S, Popp J, Frosch T. Online investigation of respiratory quotients in *Pinus sylvestris* and *Picea abies* during drought and shading by means of cavity-enhanced Raman multi-gas spectrometry. *Analyst*. 2015;140:4473–81.
44. O'Leary BM, Scafaro AP, York LM. Expanding the repertoire of plant respiration measurements. *Plant Physiol*. 2023;191:2070–83.
45. Hernández-Montes E, Escalona JM, Tomás M, Martorell S, Bota J, Tortosa I, Medrano H. Carbon balance in grapevines (*Vitis vinifera* L.): effect of environment, cultivar and phenology on carbon gain, losses and allocation. *Aust J Grape Wine Res*. 2022;28:534–44.
46. Tortosa I, Escalona JM, Opazo I, Douthe C, Medrano H. Genotype variations in water use efficiency correspond with photosynthetic traits in Tempranillo grapevine clones. *Agronomy*. 2022;12:1874.
47. Del-Saz NF, Ribas-Carbo M, Martorell G, Fernie AR, Florez-Sarasa I. Measurements of Electron Partitioning Between Cytochrome and Alternative Oxidase Pathways in Plant Tissues. In: Jagadis Gupta K, editor. *Plant Respiration and Internal Oxygen*. *Methods in Molecular Biology*. Volume 1670. New York, NY: Humana; 2017. https://doi.org/10.1007/978-1-4939-7292-0_17.
48. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing; 2023.
49. Coast O, Shah S, Ivakov A, Gaju O, Wilson PB, Posch BC, Bryant CJ, Negrini ACA, Evans JR, Condon AG, Silva-Pérez V, Atkin OK. Predicting dark respiration rates of wheat leaves from hyperspectral reflectance. *Plant Cell Environ*. 2019;42:2133–50.

50. Posch BC, Zhai D, Coast O, Scafaro AP, Bramley H, Reich PB, Ruan YL, Trethowan R, Way DA, Atkin OK. Wheat respiratory O₂ consumption falls with night warming alongside greater respiratory CO₂ loss and reduced biomass. *J Exp Bot.* 2022;73:915–26.
51. Gaju O, Bloomfield KJ, Negrini ACA, Bowerman AF, Cullerne D, Posch BC, Bryant C, Fan Y, Spence M, Stone B, Gilliham M, Furbank RT, Molero G, Pogson BJ, Mathews K, Millar AH, Pearson AL, Reynolds MP, Stroeher E, Taylor NL, Turnbull MH, Atkin OK. Accounting for the impact of genotype and environment on variation in leaf respiration of wheat in Mexico and Australia. *J Exp Bot.* 2025;76:1099–115.
52. Eustis A, Murphy KM, Barrios-Masias FH. Leaf gas exchange performance of ten quinoa genotypes under a simulated heat wave. *Plants.* 2020;9:81.
53. Escalona JM, Tomàs M, Martorell S, Medrano H, Ribas-Carbo M, Flexas J. Carbon balance in grapevines under different soil water supply: importance of whole plant respiration. *Aust J Grape Wine Res.* 2012;18:308–18.
54. Tombesi S, Frioni T, Grisafi F, Sabbatini P, Poni S, Palliotti A. The decrease of leaf dark respiration during water stress is related to leaf non-structural carbohydrate pool in *Vitis vinifera* L. *Plants.* 2022;11:36.
55. Le XH, Millar AH. The diversity of substrates for plant respiration and how to optimize their use. *Plant Physiol.* 2023;191:2133–49.
56. Fischer S, Hanf S, Frosch T, Gleixner G, Popp J, Trumbore S, Hartmann H. *Pinus sylvestris* switches respiration substrates under shading but not during drought. *New Phytol.* 2015;207(3):542–50.
57. Fan Y. From light to dark: linking the roles of mitochondria in C₄ photosynthesis with dark respiration [PhD thesis]. Canberra: The Australian National University; 2023.
58. Lambers H, Chapin FS III, Pons TL. *Plant Physiological Ecology*. 2nd ed. New York: Springer; 2008.
59. Price ER, Mager EM. Respiratory quotient: effects of fatty acid composition. *J Exp Zool Ecol Integr Physiol.* 2020;333:613–8.
60. Stare FJ, Baumann CA. The effect of fumarate on respiration. *Proc R Soc Lond B Biol Sci.* 1936;121:338–57.
61. Tcherkez G, Nogués S, Bleton J, Cornic G, Badeck F, Ghashghaie J. Metabolic origin of carbon isotope composition of leaf dark-respired CO₂ in French bean. *Plant Physiol.* 2003;131:237–44.

Tables

Table 1. Respiratory gas fluxes and respiratory quotient (RQ) across species and experimental conditions. CO₂ production was measured using IRGA and Sensirion, and O₂ consumption using PyroScience. Independent RQ (RQ_{ind}) was calculated from IRGA CO₂ fluxes and treatment-mean PyroScience O₂ consumption, whereas simultaneous RQ (RQ_{sim}) was obtained from Sensirion–PyroScience measurements. (A) C₃ and C₄ species measured at midday (MD) and nighttime (NT) (Experiment 1); (B) grapevine cultivars under contrasting water regimes and temperatures (Experiments 2 and 3). Values represent means ± SE. Different lowercase letters indicate significant differences among treatments, while asterisks indicate differences between measurement methods (*p* < 0.05). WW, well-watered; WS, water-stressed.

Species	Conditions	IRGA CO ₂ ($\mu\text{mol m}^{-2}\text{ s}^{-1}$)	Sensirion CO ₂ ($\mu\text{mol m}^{-2}\text{ s}^{-1}$)	PyroScience O ₂ ($\mu\text{mol m}^{-2}\text{ s}^{-1}$)	RQ _{ind} IRGA/PyroScience	RQ _{sim} Sensirion/PyroScience
<i>T. monococcum</i>	MD	1,37 ± 0,11 ^{abc}	0,52 ± 0,05 ^{b*}	0,81 ± 0,09 ^b	1,69 ± 0,14 ^a	0,65 ± 0,01 ^{cd*}
<i>T. monococcum</i>	NT	1,06 ± 0,06 ^{bc}	0,61 ± 0,04 ^{b*}	0,84 ± 0,04 ^b	1,25 ± 0,07 ^{abc}	0,72 ± 0,03 ^{bc*}
<i>C. quinoa</i>	MD	1,57 ± 0,18 ^a	0,96 ± 0,08 ^{a*}	1,41 ± 0,07 ^a	1,11 ± 0,13 ^b	0,67 ± 0,04 ^{cd*}
<i>C. quinoa</i>	NT	1,46 ± 0,10 ^{ab}	1,07 ± 0,15 ^{a*}	1,70 ± 0,19 ^a	0,86 ± 0,06 ^c	0,62 ± 0,02 ^{d*}
<i>S. bicolor</i>	MD	0,99 ± 0,05 ^c	0,55 ± 0,03 ^{b*}	0,68 ± 0,04 ^b	1,45 ± 0,08 ^{ab}	0,81 ± 0,01 ^{ab*}
<i>S. bicolor</i>	NT	1,07 ± 0,10 ^{bc}	0,58 ± 0,05 ^{b*}	0,70 ± 0,05 ^b	1,52 ± 0,14 ^{ab}	0,82 ± 0,02 ^{a*}
<i>V. vinifera</i> cv. Callet	WW	3,54 ± 0,12 ^a	2,02 ± 0,19 ^{a*}	2,48 ± 0,12 ^a	1,43 ± 0,05 ^{ab}	0,81 ± 0,05 [*]
<i>V. vinifera</i> cv. Callet	WS	3,17 ± 0,26 ^a	2,00 ± 0,22 ^{ab*}	2,37 ± 0,10 ^a	1,34 ± 0,11 ^{ab}	0,84 ± 0,07 [*]
<i>V. vinifera</i> cv. Manto Negro	WW	1,48 ± 0,07 ^b	1,20 ± 0,09 ^c	1,30 ± 0,11 ^b	1,14 ± 0,05 ^b	0,95 ± 0,11
<i>V. vinifera</i> cv. Manto Negro	WS	2,71 ± 0,27 ^a	1,32 ± 0,15 ^{bc*}	1,62 ± 0,17 ^b	1,67 ± 0,17 ^a	0,81 ± 0,01 [*]
<i>V. vinifera</i> cv. Grenache	WS (31 °C)	2,08 ± 0,11 ^a	1,49 ± 0,15 ^a	1,64 ± 0,12 ^a	1,27 ± 0,07	0,93 ± 0,11 ^a
<i>V. vinifera</i> cv. Grenache	WS (21 °C)	1,07 ± 0,06 ^b	0,48 ± 0,02 ^{b*}	0,71 ± 0,05 ^b	1,51 ± 0,08	0,68 ± 0,03 ^{b*}

Table 2. Putative respiratory substrates associated with apparent and absolute respiratory quotient values across species and experimental conditions. Potential respiratory substrates were assigned by comparing the observed RQ_{ind} (IRGA/PyroScience) and RQ_{sim} (Sensirion/PyroScience) with RQ values previously associated with the oxidation of different respiratory substrates in the literature. The table includes C₃ and C₄ species measured at midday (MD) and nighttime (NT) under controlled growth-chamber conditions (Experiment 1), and grapevine cultivars subjected to contrasting water regimes or measurement temperatures (Experiments 2 and 3). Substrate assignments should be interpreted as putative associations rather than direct evidence of substrate utilization. “nr” indicates that no respiratory substrate reported in the cited literature could be associated with the observed RQ value. MD, midday; NT, nighttime; WW, well-watered; WS, water-stressed.

Species / cultivar	Condition	indRQ substrate	simRQ substrate	Reference
<i>T. monococcum</i>	MD	nr	Fatty acids/Proline	[59]
<i>T. monococcum</i>	NT	Malate/fumarate	Fatty acids/Proline	[18, 59]
<i>C. quinoa</i>	MD	Succinate	Fatty acids/Proline	[55, 59]
<i>C. quinoa</i>	NT	AAs/others	Serine/Alanine	[55, 61]
<i>S. bicolor</i>	MD	nr	Aspartate	[7]
<i>S. bicolor</i>	NT	nr	Aspartate	[7]
<i>V. vinifera</i> cv. Callet	WW	nr	Aspartate	[7]
<i>V. vinifera</i> cv. Callet	WS	Malate/fumarate	Aspartate	[7, 57, 60]
<i>V. vinifera</i> cv. Manto Negro	WW	Succinate	Carbohydrates/others	[55, 58]
<i>V. vinifera</i> cv. Manto Negro	WS	nr	Aspartate	[7]
<i>V. vinifera</i> cv. Grenache	WS (31 °C)	Malate/fumarate	AAs/others	[55, 57, 60]
<i>V. vinifera</i> cv. Grenache	WS (21 °C)	nr	Fatty acids/Proline	[59]

Figures

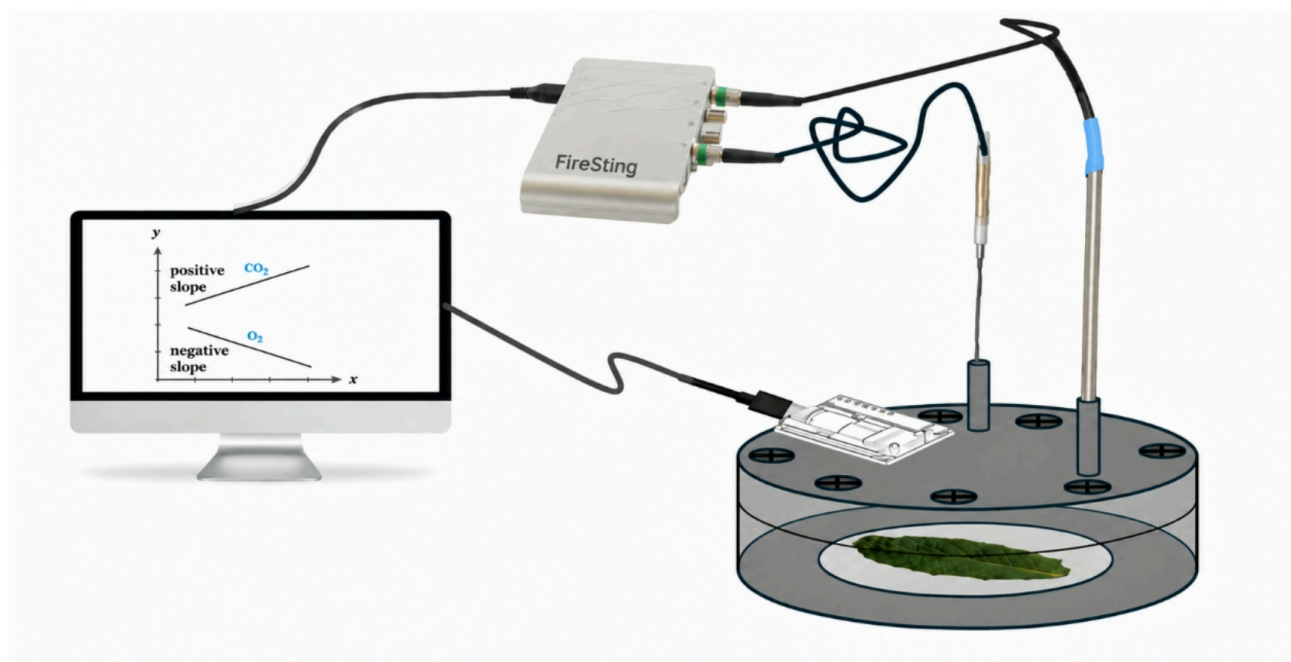


Figure 1

Schematic representation of the compact closed gas-phase system for simultaneous CO₂ and O₂ measurements. The system combines a miniaturized Sensirion CO₂ sensor, a PyroScience optical O₂ sensor, and a temperature sensor

within a sealed chamber, allowing simultaneous monitoring of CO₂ production, O₂ consumption, and chamber temperature. Changes in gas concentrations over time are recorded and displayed on a computer, with the positive slope of CO₂ concentration representing respiratory CO₂ production and the negative slope of O₂ concentration representing respiratory O₂ consumption. These simultaneous measurements are subsequently used to calculate the respiratory quotient as the ratio of CO₂ production to O₂ consumption.

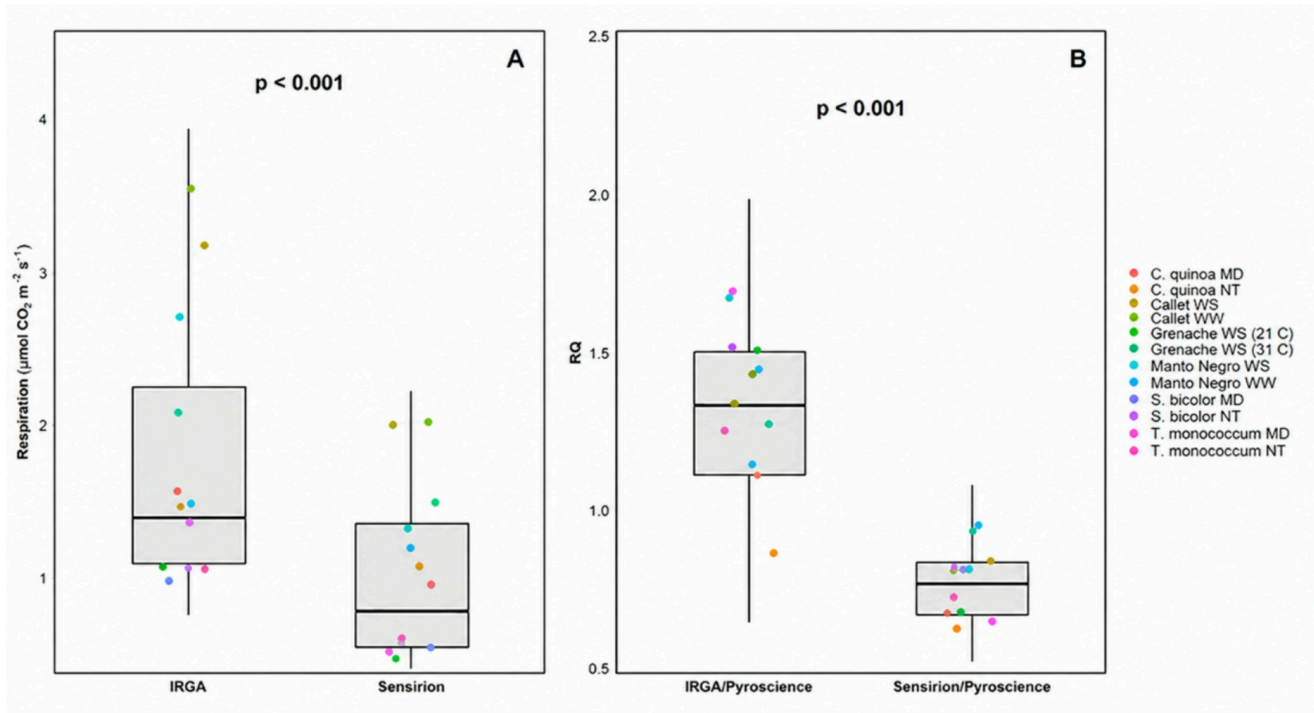


Figure 2

Comparison of respiratory CO₂ fluxes and respiratory quotient (RQ) obtained using alternative measurement approaches across all species and experimental treatments. (A) Respiratory CO₂ release measured with the conventional infrared gas analyzer (IRGA) and the miniaturized Sensirion CO₂ sensor. (B) Respiratory quotient, calculated from independently measured IRGA CO₂ production and PyroScience O₂ consumption, and RQsim, calculated from simultaneous Sensirion CO₂ production and PyroScience O₂ consumption. Colored points represent treatment mean values across the three experiments. Boxplots show the median, interquartile range, and data distribution across treatments. Differences between measurement approaches were evaluated using Student’s t-tests, with the corresponding p-values shown in each panel. MD, midday; MN, nighttime; WW, well-watered; WS, water-stressed.

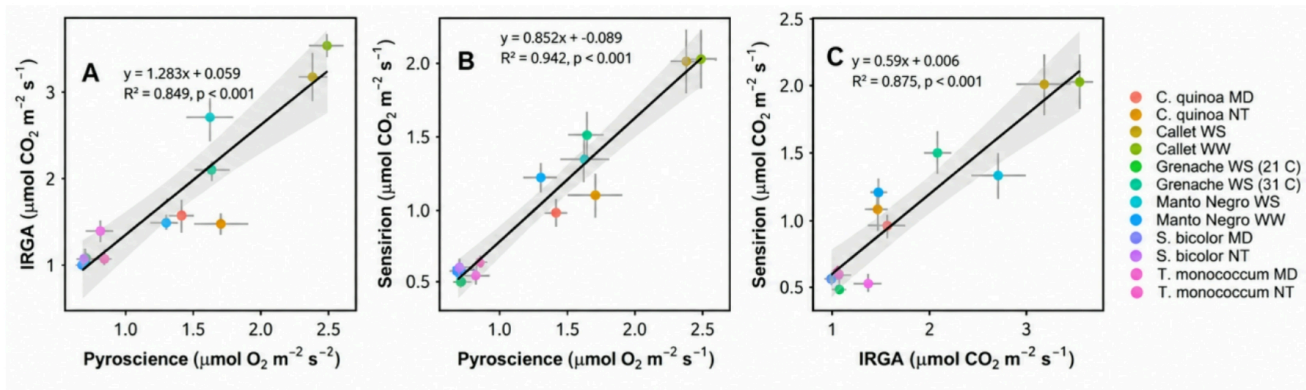


Figure 3

Relationships among respiratory CO_2 and O_2 fluxes measured using different gas-exchange systems. Linear relationships between (A) CO_2 production measured with the IRGA and O_2 consumption measured with PyroScience, (B) CO_2 production measured with Sensirion and O_2 consumption measured with PyroScience, and (C) CO_2 production measured with Sensirion and CO_2 production measured with the IRGA. Regressions were fitted using treatment mean values across all species and experimental conditions. Points represent treatment means, and horizontal and vertical error bars indicate the corresponding standard errors (SE). Solid lines represent ordinary least-squares linear regressions and shaded areas indicate the associated 95% confidence intervals. Regression equations, coefficients of determination (R^2), and p-values are shown within each panel. MD, midday; NT, nighttime; WW, well-watered; WS, water-stressed.

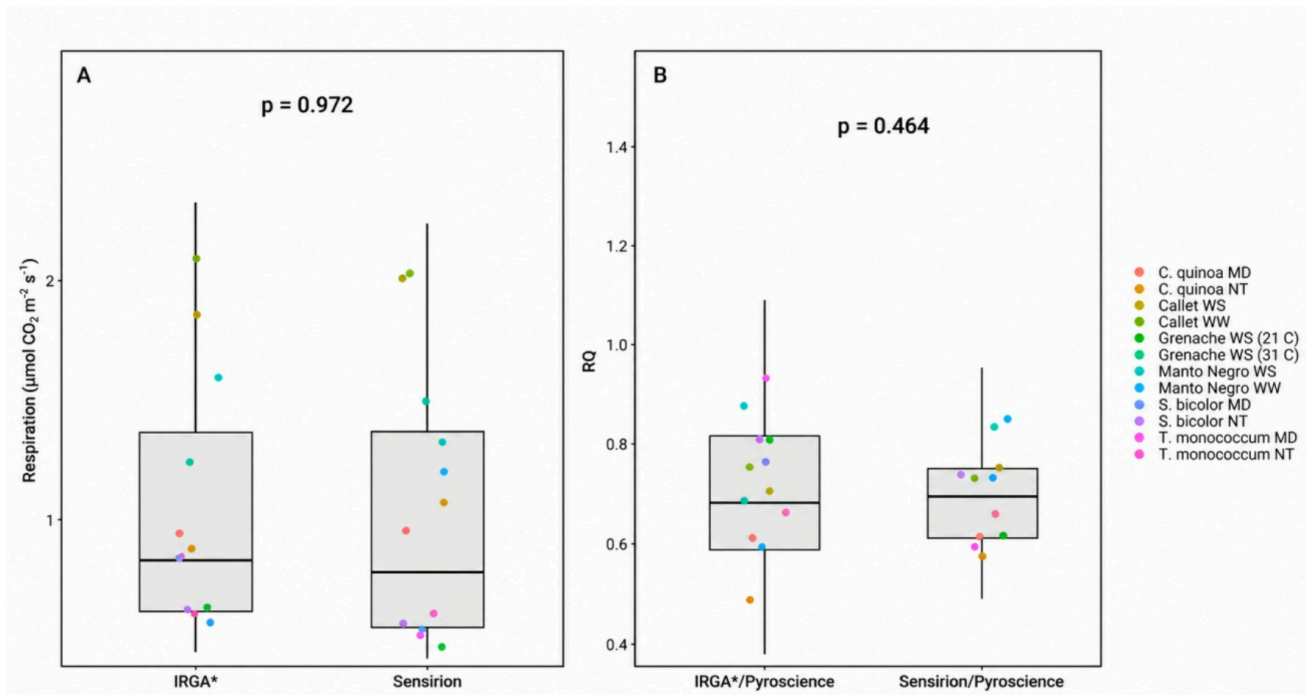


Figure 4

Effect of correcting the effective respiratory leaf area on the agreement between IRGA- and Sensirion-derived respiratory measurements. IRGA CO₂ fluxes were mathematically corrected by incorporating one-half of the leaf area covered by the chamber gasket into the effective respiratory area, accounting for the assumed contribution of CO₂ produced beneath the gasket and diffusing towards the chamber. **(A)** Comparison of area-corrected IRGA CO₂ fluxes (IRGA*) and CO₂ fluxes measured with the Sensirion sensor. **(B)** Comparison of respiratory quotient calculated using area-corrected IRGA CO₂ fluxes and PyroScience O₂ consumption (IRGA*/PyroScience) with RQ_{sim} obtained from simultaneous Sensirion–PyroScience measurements. Colored points represent treatment mean values across the three experiments. Boxplots show the median, interquartile range, and data distribution across treatments. Differences between approaches were evaluated using Student's t-tests, with p-values shown in each panel. IRGA* denotes CO₂ fluxes recalculated using the corrected effective respiratory area. MD, midday; NT, nighttime; WW, well-watered; WS, water-stressed.

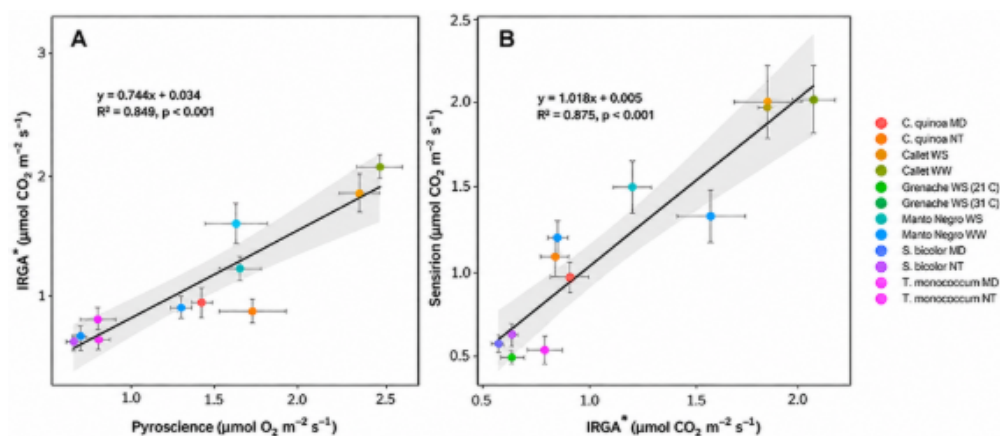


Figure 5

Relationships among respiratory gas fluxes after mathematical correction of the effective respiratory area used for IRGA measurements. **(A)** Relationship between area-corrected IRGA CO₂ production (IRGA*) and O₂ consumption measured with PyroScience. **(B)** Relationship between CO₂ production measured with Sensirion and area-corrected CO₂ production measured with the IRGA. Regressions were fitted using treatment mean values across all species and experimental conditions. Points represent treatment means, and horizontal and vertical error bars indicate standard errors (SE). Solid lines represent ordinary least-squares linear regressions and shaded areas indicate the associated 95% confidence intervals. Regression equations, coefficients of determination (R²), and p-values are shown within each panel. IRGA* denotes CO₂ fluxes recalculated using the corrected effective respiratory area. MD, midday; NT, nighttime; WW, well-watered; WS, water-stressed.

Supplementary Files

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- [Figuresupplementary1.docx](#)