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Dark Respiration Measurement from *Arabidopsis* Shoots

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[Abstract] Dark respiration refers to experimental measures of leaf respiration in the absence of light, done to distinguish it from the photorespiration that occurs during photosynthesis. Dark aerobic respiration reactions occur solely in the mitochondria and convert glucose molecules from cytoplasmatic glycolysis and oxygen into carbon dioxide and water, with the generation of ATP molecules. Previous methods typically use oxygen sensors to measure oxygen depletion or complicated and expensive photosynthesis instruments to measure CO_2 accumulation. Here, we provide a detailed, step-by-step approach to measure dark respiration in plants by recording CO_2 fluxes of *Arabidopsis* shoot and root tissues. Briefly, plants are dark acclimated for 1 hour, leaves and roots are excised and placed separately in airtight chambers, and CO_2 accumulation is measured over time with standard infrared gas analyzers. The time-series data is processed with R scripts to produce dark respiration rates, which can be standardized by fresh or dry tissue mass. The current method requires inexpensive infrared gas analyzers, off-the-shelf parts for chambers, and publicly available data analysis scripts.

Keywords: NFS1, Dark respiration, CO_2, Mitochondria, Flux

[Background] Plants produce energy in the form of ATP from glucose oxidation through mitochondrial respiration (Glucose + oxygen = carbon dioxide + water). In order to differentiate from photorespiration inherent to photosynthesis, dark respiration measurements are made in the absence of light (Graham, 1980). Mitochondria are essential organelles involved in energy production, amino acid biosynthesis, iron-sulfur (Fe-S) cluster cofactor biosynthesis, and other metabolic pathways that affect growth and stress responses (Lill, 2009). We identified two mitochondria-localized Fe-S cluster proteins, NITROGEN FIXATION S-LIKE1 (NFS1) and its interactor FRATAXIN (FH), as novel players in plant defense responses (Fonseca *et al.*, 2020). In the current manuscript, we describe the methods used in this previous research to test a possible effect of the *nfs1* and *fh* mutants, and overexpression lines, on mitochondrial respiration using measurements of dark respiration of the entire shoot and root. This simple protocol can be performed using plants grown entirely in controlled conditions in growth chambers. It involves the flux measurement of the dark respiration by-product CO_2 from tissues in small airtight chambers, using an LI-850 CO_2/H_2O gas analyzer (LICOR) in a dark room with a supplemental photosynthetically inactive yellow LED light. The CO_2 flux for each plant is measured during a 2 min 30 s interval using the software LI-8x0 v1.02 (LICOR). Specific shoot and root respiration rates (nmol CO_2

gfw^{-1} s^{-1}) are calculated from the raw CO_2 flux data using R (v.3.6.0; https://www.r-project.org/), where linear regression is used to calculate the CO_2 flux rate with the dead band set at 60 s, and then divided by total shoot fresh weight.

Prior descriptions of plant respiration measurements often included complex apparatus consisting of independent equipment to move air and measure CO_2 with an infrared gas analyzer, and using a mass flow controller (Martin *et al.*, 1981; Condori-Apfata *et al.*, 2019). More recently, photosynthesis instruments that are an order of magnitude more expensive than the gas analyzer used here have been used with custom modifications (Tomaz *et al.*, 2010). As an alternative to measuring the production of CO_2, other protocols make use of oxygen sensors to instead measure O_2 depletion (Li *et al.*, 2013). In comparison, we have accomplished the same goals using less expensive and relatively less complex off-the-shelf parts that can be assembled and used by any plant laboratory. In addition, we have made the R scripts for data analysis publicly available to ensure the reproducibility of the method.

This protocol will be useful for researchers who need to measure dark respiration as a proxy for changes in mitochondrial abundance and/or activity, as we have done in Fonseca *et al*. (2020). However, respiration has also been hypothesized to be a potential target for increasing metabolic efficiency, as has been done for photosynthesis (Amthor *et al.*, 2019). Simulation results highlight the opportunity to target respiration as an unappreciated avenue for crop improvement, where both leaf and root respiration need to be considered for the whole-plant carbon allocation analysis (Holland *et al*., 2020). Indeed, a similar protocol as outlined here was used to measure in-light root respiration of 1,104 wheat seedlings to conduct a genome wide association study (Guo *et al.*, 2021). This work found substantial heritable variation for specific root respiration as well as candidate genes responsible for that variation. Bunce (2021) showed that CO_2 depletion methods for respiration closely match the total mass reduction estimates of respiration, validating the approach. Therefore, this reproducible protocol can have an impact on multiple fields of plant biology, ranging from understanding the molecular basis of respiration to study respiration as a breeding target.

Materials and Reagents

1. Falcon 6-well clear flat bottom plate (Corning, catalog number: 353224)
2. Petri plates (15 × 100 mm) (Carolina, catalog number: 741251)
3. 7.5 cm^3 internal volume opaque PVC pipe with threaded ends (United States Plastic Corp, 1/4" × 6" Schedule 80 CPVC Nipple, catalog number: 30207)
4. Threaded cap with 1/8” hole drilled in the center (United States Plastic Corp, 1/4" FNPT Nylon Threaded Cap, catalog number: 62164)
5. Quick-connect male and female fittings (LI-COR, Inc., catalog numbers: 300-07124 and 300-07125)
6. Balston filter (LI-COR, Inc., catalog number: 300-01961)
7. Rubber seals (LI-COR, Inc., catalog number: 167-07256)
8. Parafilm M laboratory film (Amazon, catalog number: PM999)

9. Bev-A-Line 1/4" OD 15 m tubing (LI-COR, Inc., catalog number: 8150-250)
10. Quick connect, female bulkhead fitting (LI-COR, Inc., catalog number: 300-07126)
11. Quick connect, plug bulkhead (LI-COR, Inc., catalog number: 300-07127)
12. Quick connect straight union (LI-COR, Inc., catalog number: 300-03123)
13. Aluminum foil (Amazon, any brand)
14. Drierite (LI-COR, Inc., catalog number: 622-04299)
15. *Arabidopsis* seeds (ABRC., https://abrc.osu.edu)
16. Murashige & Skoog (MS) media with vitamins (Grainger, catalog number: M70800-50.0)
17. Sucrose (VWR, catalog number: BDH9308-500G)
18. Potassium hydroxide (KOH) (VWR, catalog number: BDH9262-500G)
19. Phytagel (Sigma-Aldricht, catalog number: 71010521)
20. Ethanol absolute 100% (VWR, catalog number: 71006012)
21. Triton X-100 (Fischer scientific, catalog number: AC327372500)
22. Sodium Hypochlorite 8% (Clorox Disinfecting Bleach, catalog number: CLO32260)
23. 1/2 MS liquid media pH 5.7 (see Recipes)
24. Bleach solution (see Recipes)

Equipment

1. Orbital Shaker (Thermolyne Bigger Bill, catalog number: M49235)
2. LI-850 CO_2/H_2O Analyzer (LI-COR, Inc. catalog number:)
3. Computer with Windows 10 and USB ports to run LI-COR software
4. Bead bath (Lab Armor, catalog number: 74300-714)
5. Sterile cabinet (Thermo Scientific, catalog number: 13-261-221)
6. Autoclave (Steris Amsco Century, catalog number: SG-120)
7. Metallic beads (Lab Armor, catalog number: 42370-004)
8. Timer/stopwatch (Big Digit Timer, catalog number: ML5004)
9. Compressed air duster (Blow Off Duster, catalog number: 152-112-226)
10. Laboratory balance scale (Mettler Toledo, catalog number: ME103TE)
11. Curved tip forceps for plant handling (Epredia, catalog number: 1631)
12. Scissors for plant handling (Amazon, any brand)

Software

1. LI-8x0 v1.02 (LI-COR, Inc., https://www.licor.com/env/support/LI-850/software.html)
2. R studio (R Core Team, https://www.r-project.org/)

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Procedure

A. Seedling growth

1. First, sterilize *Arabidopsis* seeds by adding 100% ethanol to seeds in an Eppendorf tube for 1 min and inverting the tube to mix. Pipette in 1 ml bleach solution (see Recipes). Mix by inversion for 5 min. Remove bleach solution and resuspend seeds in sterile dH_2O, mixing for 1 min. Repeat the wash with dH_2O three more times and resuspend seeds in sterile 0.01% agar. Spread seeds in a Petri plate containing solid one-half strength MS-phytagel media. Move plates to a dark cold room (4°C) to stratify seeds for 2 days. Place plates under light for 5 days to allow germination to occur. Transfer germinated seedlings to a Falcon 6-well culture plate, with each well containing around 30 ml liquid one-half strength 1/2 MS liquid media (see recipe). Before measurements, allow seedlings to grow (22°C, 12 h light/dark) for an additional 18 days in an orbital shaker (placed inside the plant growth room) at slow speed (Figure 1).

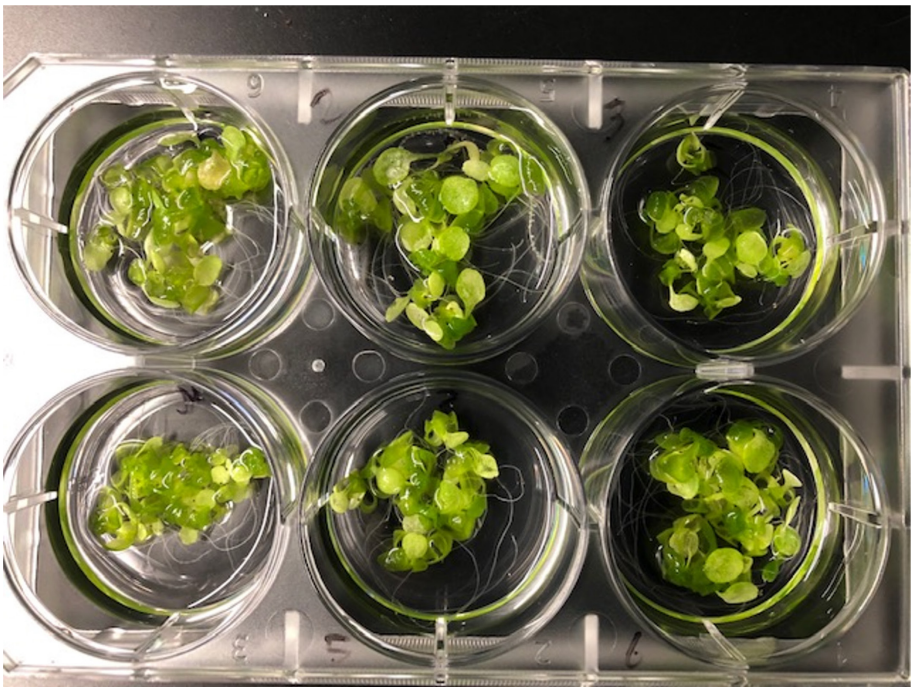

Figure 1. Seedling growth in 1/2 MS liquid media after 23 days (12 h light/dark)

B. Respiration measurement preparation

1. Assemble the light- and airtight custom respiration chamber by attaching the threaded PVC pipe to the quick-connect fittings sealed with the rubber seals. Connect the Balston filter between the sample and the LI-850 in-flow direction using straight unions. The bulkhead connectors go on the ends of tubing that connect to the LI-850 (Figure 2). Check the connection to make sure that there is no air leakage by submerging in water and checking for air bubbles, or gently blowing on the chamber and checking that the CO_2 levels in the chamber do not increase. Use Parafilm or petroleum jelly to fix any leaks that may interfere with instrument measurements.

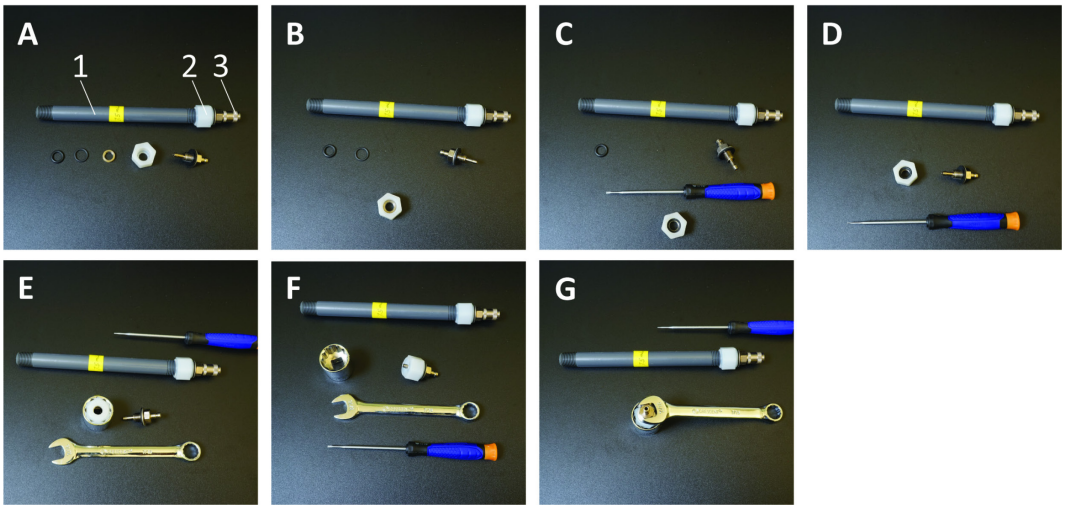

Figure 2. Custom respiration chamber assembly. (A). Custom respiration chambers comprised of a (1) threaded PVC pipe and (2) threaded cap with (3) quick-connect fittings sealed with rubber seals. (B-G). Close-up view showing the sequential assembly order of the male quick-connect fitting.

2. Power on the bead bath and set to 23°C and allow time for the temperature to become uniform.
3. Power on LI-850 and connect to a computer USB port. Open “LI-8x0” software and allow the machine to warm up to an operating temperature of 51°C (approximately 10 min). Choose data logging rate of one reading per second and a tab-delimited .csv file output type.
4. With the chamber empty, connect it to the LI-850, bury the chamber in the bead bath (Figure 3), type in a filename for the control reading in the LI-850 software, and then press “Start” to log CO_2 concentration in the chamber. Start timer and log for 2 min 30 s before pressing “Stop” logging (Figure 4). This should generate a stable CO_2 concentration as a check to ensure the equipment is set up correctly (horizontal line on the live time by CO_2 graph).

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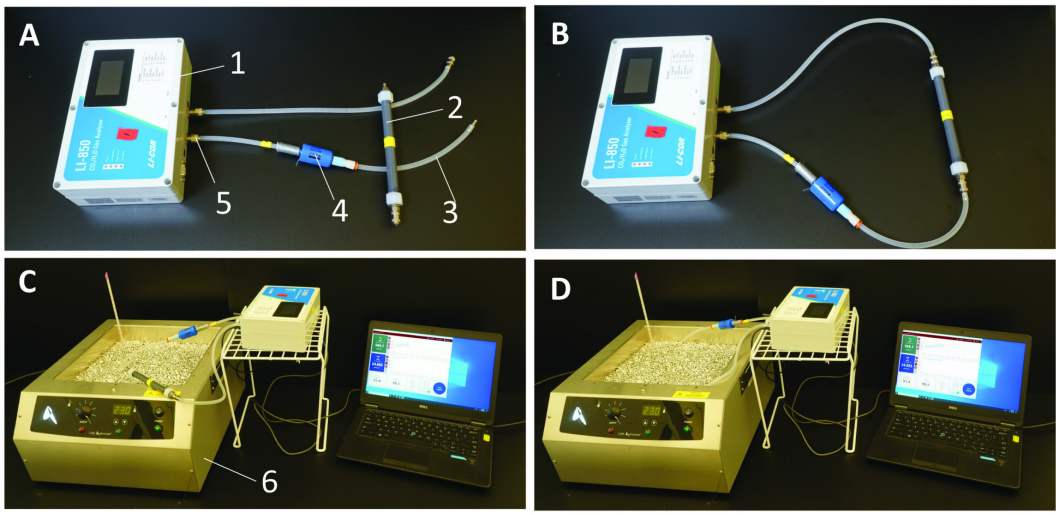

Figure 3. LI-850 and custom respiration chamber assembly. (A) The LI-850 is connected by USB (1) and data logged to a laptop running the "LI-8x0" software. The chamber (2) is connected by tubing (3) to the LI-850. A Balston filter (4) is fitted between the chamber and the LI-850 in-flow direction using straight unions. Bulkheads (5) go on the ends of tubing that connect to the LI-850. (B) Chamber connected to the LI-850. (C and D) For the respiration measurements, the chamber cap is removed and the sample placed inside. The chamber is then closed and buried in a bead bath (6).

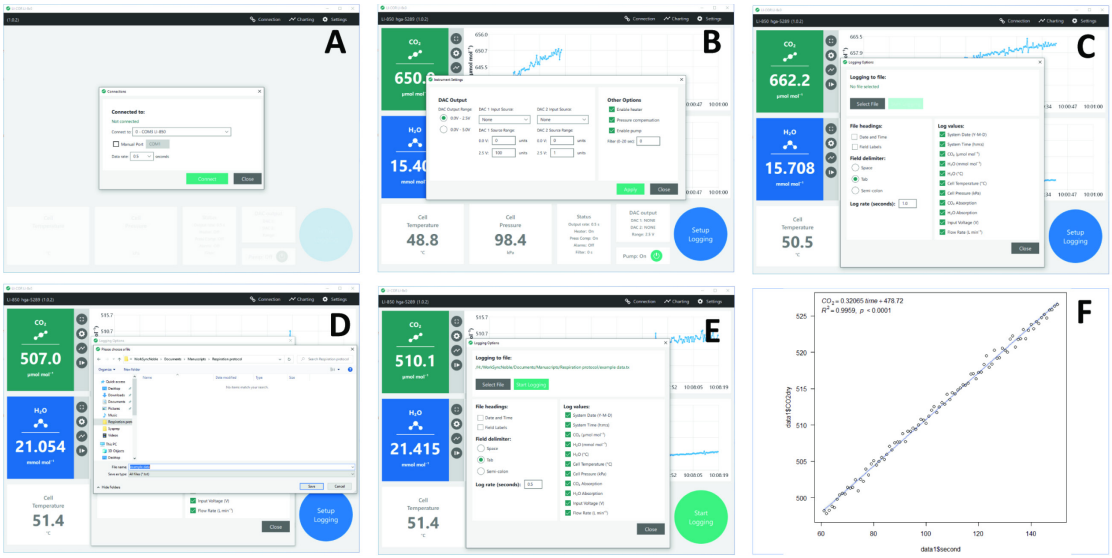

Figure 4. Shoot respiration data collection and analysis. (A) Connect the LI-850 to a laptop running the "LI-8x0" software. (B) Select instrument default options. (C and D) Select file output readings. (E) Save file and start measurements. (F) Exported LICOR.txt files are then batch processed in R to calculate the rate of CO_2 accumulation in the chamber and, therefore, of respiration.

C. Fresh biomass and dark respiration data collection

1. Wrap an entire 6-well culture plate containing the growing plants in aluminum foil for 1 h before CO_2 flux measurements. For shoot dark respiration, operate in a dark room with a supplemental photosynthetically inactive yellow LED light.
2. Carefully sever and separate the shoot material from the root material. Record the fresh weight of shoots using a scale before transferring them into a light- and airtight chamber described in Step B1 (see Table S1 for examples of fresh weights recorded).
3. After recording the fresh weight (Step B1), connect the chamber containing shoot material to the LI-850 immediately. Type in the sample name into the LI-850 software and press "Start" to log CO_2 concentration in the chamber. Start timer and record CO_2 data for 2 min 30 s before pressing "Stop." A detectable accumulation of CO_2 in the chamber should be observed during this time if the equipment is set up correctly (positive slope on the live time by CO_2 graph). As the shoot material has been severed from the whole plant, respiration measurements longer than 10 min are not recommended.
4. Disconnect the chamber from the LI-850 and use a compressed air duster to remove all plant material from the chamber. Connect the LI-850 to a chamber filled with Drierite for at least one minute between samples to remove any moisture accumulated. Ensure that the LI-850 live CO_2 readings have returned to atmospheric levels before connecting the next sample.
5. Repeat steps 5 and 6 with the remaining samples.
6. One ".TXT" file containing the raw CO_2 concentration measurements over time will be generated per sample. Transfer all generated LI-850 output files to a folder for data analysis.

Data analysis

1. In R, run the "LI850_respiration.R" code file (see supplemental document) and set the R working directory to a folder containing a copy of the generated LI850 ".TXT" files and a shoot fresh weight file containing all shoot weights "ShootFreshWeight.CSV."
2. Optionally, change the dead band value in the code from the default parameter of 60 s to filter out values before the respiration measurement stabilizes. Here, the first 60 s of the 2 min 30 s reading was removed as the LI-850 measurements CO_2 flux rate stabilized.
3. Follow through the code to calculate specific shoot respiration rates (nmol CO_2 gfw^{-1} s^{-1}) in batch for all samples in the folder. Specific shoot respiration rates are calculated using a linear regression of the CO_2 flux rate, and then the value is divided by total shoot fresh weight (see Table S1).
4. Use the forward and back arrows in the plots pane of RStudio to browse through the linear regression graphs for each sample and ensure all are expected with a positive and tight slope.
5. Save the processed data as a ".CSV" file.

Notes

This protocol is best performed by two people over a period of several hours. In our case, it took 3-4 h to process 10 samples with 5-6 replicates. In our experimental setup, one researcher handled the samples and measured fresh weight using a balance while the other handled the CO_2 flux measurements using the LI-850. Additional researchers operating multiple LI-850 machines can increase the sample throughput. This protocol can also be broadly applied to other tissue types, such as plant roots, for measuring respiration rates.

Recipes

1. 1/2 MS media pH 5.7
   Murashige & Skoog (MS) media with vitamins 2 g
   Sucrose 3 g
   dH_2O 800 ml
   Adjust pH to 5.7
   Fill container up to 1 L with dH_2O
   Add 8 g of Agar if preparing solid media at this point
   Autoclave at 121°C for 15 min
2. Bleach solution
   1:1 bleach and dH_2O
   0.02% Triton X-100

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Competing interests

The authors declare no financial and non-financial competing interests.

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