Respiration Traits as Novel Markers for Plant Robustness Under the Threat of Climate Change: A Protocol for Validation

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Abstract

Respiration traits allow calculating temperature-dependent carbon use efficiency and prediction of growth rates. This protocol aims (1) to enable validation of respiration traits as non-DNA biomarkers for breeding on robust plants in support of sustainable and healthy plant production; (2) to provide an efficient, novel way to identify and predict functionality of DNA-based markers (genes, polymorphisms, edited genes, transgenes, genomes, and hologenomes), and (3) to directly help farmers select robust material appropriate for a specified region. The protocol is based on applying isothermal calorespirometry and consists of four steps: plant tissue preparation, calorespirometry measurements, data processing, and final validation through massive field-based data.

The methodology can serve selection and improvement for a wide range of crops. Several of them are currently being tested in the author’s lab. Among them are important cereals, such as wheat, barley, and rye, and diverse vegetables. However, it is critical that the protocol for measuring respiration traits be well adjusted to the plant species by considering deep knowledge on the specific physiology and functional cell biology behind the final target trait for production. Here, *Daucus carota* L. is chosen as an advanced example to demonstrate critical species-specific steps for protocol development. Carrot is an important global vegetable that is grown worldwide and in all climate regions (moderate, subtropical, and tropical). Recently, this species is also used in my lab as a model for studies on alternative oxidase (AOX) gene diversity and evolutionary dynamics in interaction with endophytes.

Key words Calorespirometry, Predicting plant robustness, Yield stability, Temperature tolerance, Climate change, Biomarker, Functional marker, Genomics, Hologenomics, *Daucus carota* L.

1 Introduction

Calorespirometry was developed as a means for understanding metabolic adaptation and acclimation to environmental conditions [1]. Nevertheless, it was already early suggested that the methodology could be helpful in breeding programs to improve temperature-dependent growth performance [2]. However, despite this early insight, the concept has never been applied on important crops in global breeding or for farmers in order to select...
the better material for their region. More recently, the basic concept of this approach got significant support through new knowledge on the significance of mitochondria and specifically on alternative respiration for managing plants’ adaptive multi-stress responses (reviewed in [3] and [4]) combined with deep phenotyping initiatives in plant breeding [5–7]. Alternative oxidase (AOX) genes have been proposed as promising candidates for functional marker development linked to temperature responses and growth performance [8–11]. In carrot, applying the present protocol to root meristems in a phenotyping assay with a small set of inbred lines, it was possible to conclude that calorespirometry might be used to identify genotype-specific optimum temperatures and low temperature limits for root biomass growth [12]. Recently, it was also found that early AOX expression during reprogramming of quiescent carrot tap root tissue to cell division-based growth coincided with a critical time point for biomass prediction measured by calorespirometry [10].

Calorespirometry is capable of measuring temperature-dependent metabolic heat rates and near-simultaneous CO₂ production rates in small amounts of growing plant tissue. CO₂ production rates can be obtained by capturing tissue-emitted CO₂ as bicarbonate through temporarily added NaOH in an exothermic reaction (enthalpy change: \( \Delta H = -108.5 \text{ kJ/mol} \)). This allows calculating overall temperature-dependent substrate carbon conversion efficiency and structural biomass formation rates. The calculation is based on thermodynamic modeling explained by Hansen et al. [1, 13]. It takes into account an enthalpy balance model valid under mainly aerobic conditions where the energy released by a respiring tissue is equal to the sum of energy from catabolic reactions plus that absorbed by anabolic reactions [1, 14, 15].

In carrot, yield stability depends crucially on the regulation of the central root meristem that determines secondary growth of the tap root, which is the harvest organ ([16] and references therein). Shoot growth is not critically limiting root growth when plants are growing under optimal abiotic and biotic conditions. However, under difficult climate conditions due to extreme changes between low and high temperatures and under weed pressure for competitive plant growth, temperature-dependent rapid seedling and shoot growth becomes critical for yield stability. Thus, a methodology that is able to predict growth performance in both root and shoot can serve as a valuable tool in carrot pre-breeding.

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2 Materials

2.1 Plant Source Material

Carrot seedlings and plants for shoot and secondary root sampling (see Subheading 3.1): Genotypes should be selected from breeding material or registered varieties with known environmental responses across high numbers of locations and years.
2.2 Equipment

Calorespirometric measurements can be made in a model 4100 Multi-Cell Differential Scanning Calorimeter (TA Instruments, USA). This instrument has four 1-mL ampoules, three for samples and one ampoule acting as a reference. Heat emitted by material inside the ampoules is measured by the equipment as heat rates. The calorimeter is permanently connected to a water bath for temperature control. A nitrogen container/bottle is connected to the calorimeter in case temperatures will be measured below 15 °C in order to keep the atmosphere in the equipment dry (see Note 1).

2.3 Laboratory Material

1. Gloves, sharp laboratory knifes, and forceps are required for carrot material sampling.
2. Tubes for preparing fresh 0.4 M NaOH solution (see Note 2).
3. Tips of plastic micro tubes are cut from normal tubes to a size that allows placing them into the ampoules. They are used as containers for 40 μL of 0.4 M NaOH solution for CO2 capturing. Even when placed in a completely horizontal way, the liquid will stay inside.
4. Laboratory nitrogen gas in a container/bottle connected to the calorimeter.

3 Methods

3.1 Preparation of Carrot Samples from Root Tissue and Shoots

3.1.1 Collecting Root Meristem from Tap Roots

Grow seeds that had been stored at cool temperatures (~4 °C) in pots with commercial substrate or into sandy soil (see Note 3). Depending on maturity characteristics of the cultivar, sample carrot plants from the moment when the root meristem is well developed, meaning when root length growth is finalized and secondary root growth is taking place (e.g., 3–4 months after seeding). Manually separate the tap root meristem circle as a layer of ten cells (thickness) around the central xylem from xylem and secondary phloem and cut it into smaller pieces in order to fit into the calorimeter ampoules. Take samples of ca. 200 mg of meristem for each measurement.

3.1.2 Collecting Shoot Material

Sow seeds from selected genotypes on watered paper. In the seedling stage, when both cotyledons are developed, transfer seedlings to soil. When 5–10 leaves have emerged, sample leaf material (see Note 4).

3.2 Calorespirometry

3.2.1 Measurements

Calorespirometry measurements on carrot samples follow the general protocol given by Hansen et al. [1] and the specific protocols developed for carrot samples by Nogales et al. [12, 17]:
1. Place carrot root tissue or shoot samples into one of the three ampoules for heat rate (Rq) measurements (see Subheading 2.2) and run samples at a series of temperatures in isothermal mode.
ranging normally between 5 and 40 °C. Take endpoint measurement values and subtract from each measured value a baseline value (see Note 5) obtained from measurements with empty ampoules. Temperature changes are run from less stressful to more stressful conditions (see Note 6). For each temperature change, program an equilibrium time of 600 s (s) before starting measurements.

2. After the Rq signal has become stable during about 300 s, record the first endpoint measurement value; normally this can be obtained after around 35 min (see Note 7).

3. Open sample ampoules, introduce a vial of 0.4 M NaOH solution in order to capture emitted CO2 from the plant tissue at the measurement temperature, and close the ampoule again. Record the heat rate after it has become stable again for around 300 s and take the second measurement value.

4. Remove the NaOH vial from the ampoules in order to measure again the heat rate and take the third measurement endpoint value when heat rates have become stable (see Note 8). Perform measurements in repetitions in the same way with at least three independent samples.

3.2.2 Data Processing
(See Note 9)

1. Calculate the mean of the measured heat rates (Rq) before and after adding NaOH (3.2.1 points 2 and 4) (unit: μW or μJ/s).

2. Subtract this mean heat rate from the measured Rq in the presence of NaOH (unit: μJ/s) to obtain Rq that relates to CO2 production (Subheading 3.2.1, step 3).

3. Calculate the rate of CO2 production (RCO2) by dividing the mean Rq (Subheading 3.2.2, step 1) by the negative of the enthalpy change, i.e., by 108.5 μJ/nmol (unit: nmol/s).

4. Calculate the calorespirometry ratio Rq/RCO2 (unit: μJ/nmol or kJ/mol CO2) by using the Rq value obtained under Subheading 3.2.2 step 2, and the value for the rate of CO2 production obtained under Subheading 3.2.2, step 3 (see Note 10).

5. Calculate substrate carbon conversion efficiency (ε) in two steps from calorespirometry ratios (Rq/RCO2) that are lower than 470 kJ/mol CO2 (see Subheading 3.2.2, step 4, and Note 10):
   (a) Calculate a “factor x”: \([Rq/RCO2-470]/-30\) (see Note 11).
   (b) Calculate ε by dividing “factor x” (a) by its value plus 1 (no unit) (see Note 12).

6. Calculate the rate of biomass formation (Rbiom): “factor x” times RCO2 (see Subheading 3.2.2, steps 5a and 3) (no unit) (see Note 13).
7. Analyze the temperature dependency of both values (ε and Rbiom) by temperature response curves (x-axis: temperature; y-axis: ε or Rbiom) and use these graphs as a basis to identify differences between genotypes in terms of its stable or unstable response across the tested temperatures *(see Note 14)*.

8. Apply common statistics methodologies to identify significant differences between genotypes at defined temperatures or for the temperature response curve considering at least three independent repetitions.

### 3.2.3 Validation

1. Validation depends on the association between ε and Rbiom of roots and shoots with yield stability at a satisfactory level of product quality and yield. Compare results achieved by this protocol to already available large data from field experiments. Data from many locations and different years can be obtained from breeders or by variety registration catalogues. In case the ranking of varieties or breeding material for robustness/yield stability obtained from field trials could have been predicted by ε and/or Rbiom, these respiration traits can be validated as useful biomarkers for plant selection in carrot pre-breeding.

2. In case of a positive validation, the protocol can also be used to select carrot varieties as a service to farmers. Local temperature distribution curves, temperature-dependent climate changes, predicted optimum temperature response, and temperature limits for respiration and growth performances must be considered.

### 4 Notes

1. To avoid water condensation, the measuring chamber should never be opened unless the temperature is above 15 °C.

2. NaOH solution can be stored and used for short periods of around 3 weeks depending also on how often and long it will be opened during handling. For a new trial, fresh 0.4 M NaOH should be prepared.

3. The exact conditions for carrot growth in pots (greenhouse or growth chamber) are considered not important as long as all genotypes for screening are grown in the same location under comparable abiotic and biotic conditions.

4. The number of leaves taken for the measurement depends on their size and the heat produced *(see Subheading 2.2)*. Before putting the leaves into the ampoules they are shortly dipped in 70% ethanol, washed twice with sterile aqua dest., and then surface-dried by using a paper towel.

5. Baselines need to be taken for each ampoule, since the values will differ depending on the ampoules and equipment conditions.
6. It is important to avoid beginning with stressed tissue that might interfere with subsequent measurements. Before the first measurement is started, it is also recommended to allow an adaptation time of around half an hour at a non-stressful temperature close to the first measurement temperature.

7. Stressed plant material that might show a rapid decrease in heat rate will not give valid measurement values. On the contrary, contamination by rapidly growing microorganisms might also not result in valid measurement values because of increasing heat rates.

8. A typical aspect of the isothermal curves minus/plus/minus NaOH is given in Fig. 1.

9. An example for a step-by-step calculation is given in Table 1.

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**Fig. 1** A typical aspect of isothermal curves minus/plus/minus NaOH

**Table 1**

Step-by-step data processing to obtain carbon use efficiency and rate of biomass formation

| Example for data from carrot shoot (cv. Rotin): Measured Rq values (Subheading 3.2.1) | Minus NaOH (1) | Plus NaOH (2) | Minus NaOH (3) |
| --- | --- | --- | --- |
| 83 | 108 | 80 |

Steps of data processing (Subheading 3.2.2)

| Step | Value |
| --- | --- |
| 1. | 81.5 |
| 2. | 26.5 |
| 3. | 0.244 |
| 4. | 333.69 |
| 5.a | 4.54 |
| 5.b | 0.82 |
| 6. | 1.11 |
10. The ratio, $R_q/RCO_2$, is inversely related to the efficiency by which a plant system is using its available energy for biomass growth. Heat released per mole consumed oxygen in living material is called “oxycaloric equivalent” (e.g., [18]). Thornton’s rule states that this value is deduced from the oxidation state of carbon and can be obtained by chemical reaction through combustion of organic compounds. It reaches values for $\Delta H_{O2}$ between $-430$ and $-480$ kJ/mol with an average value of $-455 + / -15$ kJ/mol. From carbohydrates every C-mole releases 470 kJ/mol, a typical fatty acid provides 650 kJ/mol, lipids 611 kJ/mol, and protein is expected to release around 543 kJ per mole [1, 19]. The applied model assumes carbohydrate as the main component in the growing biomass and aerobic metabolism [1]. The amount of energy characterizes the quantity of heat lost from the tissue per mole of CO$_2$ produced. Thus, it can be expressed by the ratio $R_q/RCO_2$. In growing tissue of plants, a measured value equal to 470 kJ/mol indicates under these assumptions that no growth takes place, which is equal to zero substrate carbon conversion efficiency. Values $<470$ kJ/mol indicate efficiencies $>0$ and values higher than 470 kJ/mol indicate loss of energy for biomass growth through the usage of reduced substances for substrate oxidation. Thus, values for efficiency lie always between $>0$ and $<1$.

11. This step can only be applied to calculate efficiency values $>0$, meaning the ratio $R_q/RCO_2$ needs to be $<470$ kJ/mol. Considering that the elemental composition and consequently heat of combustion of all growing plant material are approximately the same [1, 20, 21], it is assumed in the model that the change of enthalpy ($\Delta H_b$) will be $+30$ kJ mol$^{-1}$ °C. Then $R_q/RCO_2$ can be set equal to 470–$30 \[ \varepsilon/1-\varepsilon \] [9]. Factor $x = \varepsilon/[1-\varepsilon]$.

12. $\varepsilon$ can be calculated from measured $R_q/RCO_2$ values and the calculated factor $x$:

$$\varepsilon = x/(x + 1).$$

13. According to the model, the rate of biomass formation (Rbiom) can only be calculated when $R_q/RCO_2$ is $<470$ kJ/mol (see Note 8). Rbiom is equal to growth rate. Following Hansen et al. [1, 13] respiration-driven growth of structural biomass ($C_{biomass}$) can be described as

$$C_{substrate} + \text{NPK etc.} + xo_2 \rightarrow \varepsilon C_{biomass} + (1-\varepsilon) CO_2.$$

Herein $\varepsilon$ is the fraction of substrate carbon passing through respiration and being used in anabolism to form structural biomass. $(1-\varepsilon)$ is the fraction of substrate carbon lost as CO$_2$. By definition $\varepsilon$ is called carbon use efficiency or substrate
carbon conversion efficiency. \( \text{Rbiom}/\text{RCO}_2 \) is equal to \( \frac{\varepsilon}{(1-\varepsilon)} \); thus \( \text{Rbiom} \) is equal to the product of \( \text{RCO}_2 \) times “factor \( x \).”

14. Small differences in \( \varepsilon \) will have high effects on growth over time [13].

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