

Rice Root Organic Acid Efflux Measurement by Using Ion Chromatography

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[Abstract] Organic acids secreted from plant roots play important roles in various biological processes including nutrient acquisition, metal detoxification, and pathogen attraction. The secretion of organic acids may be affected by various conditions such as plant growth stage, nutrient deficiency, and abiotic stress. For example, when white lupin (*Lupinus albus* L.) is exposed to phosphorus (P)-deficient conditions, the secretion of citrate acid from its proteoid roots significantly increases (Neumann *et al.*, 1999). This protocol describes a method for the collection and measurement of the efflux of organic acids (oxalate, malate, and citrate) from the roots of rice cultivar Nipponbare ('Nip') under different nitrogen forms (NH_4^+ and NO_3^-), together with different P supply (+P and -P) conditions.

Keywords: Organic acids, Rice, Collection, Measurement, Ion chromatography

[Background] In addition to enzymatic methods (Delhaize *et al.*, 1993) and high-performance liquid chromatography (HPLC) (Chen *et al.*, 2013), ion chromatography is another widely used method for the determination of organic acids, which has previously been employed to detect the significant increase in oxalate content in taro root exudates during Al^{3+} stress (Ma and Miyasaka, 1998). Compared to ion chromatography, alternative methods have their own defects. For example, enzymatic methods require the use of enzymes that can easily undergo denaturation. Moreover, it is difficult to distinguish oxalate acid from Cl^- peaks by HPLC. Here, we describe a method for analyzing organic acids secreted by rice roots using ion chromatography. This method could be used in the analysis of organic acids that are secreted by other hydroponically cultivated plants.

Materials and Reagents

1. 0.2 μm syringe filter (Beyotime, catalog number: FF252)
2. Rice seedlings
3. Nutrient solution
4. Amerlite IR-120B resin (H^+ form) (Alfa Aesar, catalog number: L14285)
5. Dowex 1 x 8 resin (100-200 mesh, formate form)
6. Distilled water
7. Hydrochloric acid (HCl) (Sigma-Aldrich, catalog number: 258148)
8. Formic acid (HCOOH) (Sigma-Aldrich, catalog number: 695076)
9. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: 221465)

10. Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (Sigma-Aldrich, catalog number: 223506)
11. Oxalate (Sigma-Aldrich, catalog number: 75688)
12. Malate (Sigma-Aldrich, catalog number: M8304)
11. Citrate (Sigma-Aldrich, catalog number: 251275)
12. 1 N HCl (see Recipes)
13. 2 N HCl (see Recipes)
14. 2 M HCOOH (see Recipes)
15. 4 N NaOH (see Recipes)
16. 0.5 mM CaCl_2 (see Recipes)

Equipment

1. 1.25 L black plastic pot (plastic pot must be opaque, if required, wrap it in black tape)
2. pH meter (Mettler Toledo, model: S40/SG78/SG23/SG68)
3. Cation exchange column (16 mm x 14 cm) (Bio-Rad Laboratories, model: 732-1010)
4. Rotary evaporator (IKA, model: RV10)
5. Ion chromatography (Thermo Fisher Scientific, Dionex, model: ICS 3000 System)
6. IonPac AS11 anion-exchange analytical column (4 x 250 mm)
7. Guard column (4 x 50 mm)

Procedure

1. Cultivate rice seedlings in full strength nutrient solution for 7 d. Afterwards, transplant 10 uniform seedlings to 1.5-L pots. After 6 d of treatments, discard the nutrient solution, and treat these rice seedlings with 0.5 mM CaCl_2 (pH 5.5) overnight to collect the organic acids (Figure 1).

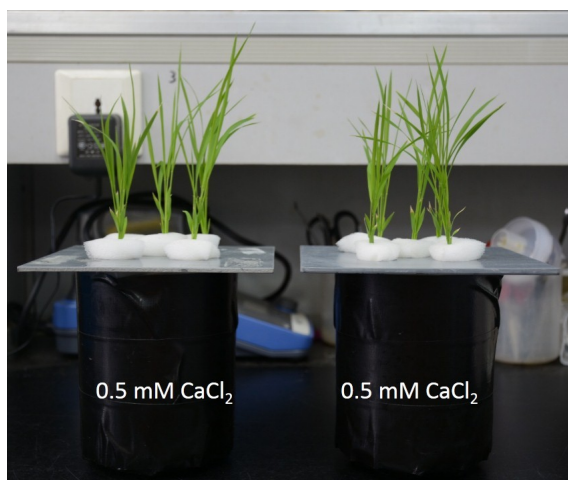


Figure 1. The apparatus for collection of root exudates

- After 12 h, collect and weigh the fresh roots of the treated rice seedlings. Pass the root exudates in the 0.5 mM CaCl_2 solution through a cation exchange column (16 mm x 14 cm) that contained 5 g of Amerlite IR-120B resin (H^+ form) and an anion exchange column filled with 1.5 g of Dowex 1 x 8 resin (100-200 mesh, formate form) (Figure 2). Overall, this process takes approximately about 5.5 h.

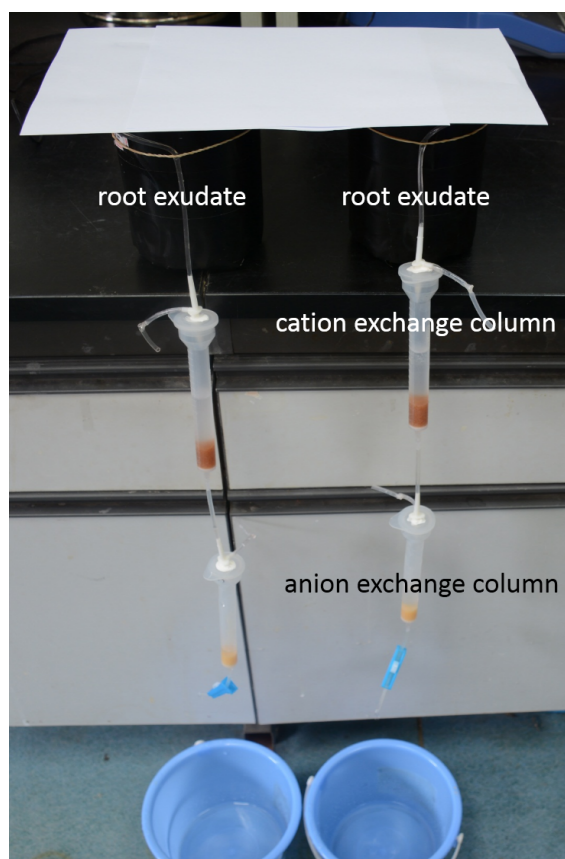


Figure 2. The equipment for retention of the organic acids

- Elute the organic acid anions that were retained in the anion exchange resin immediately after the second step using 15 ml of 1 N HCl (Figure 3).

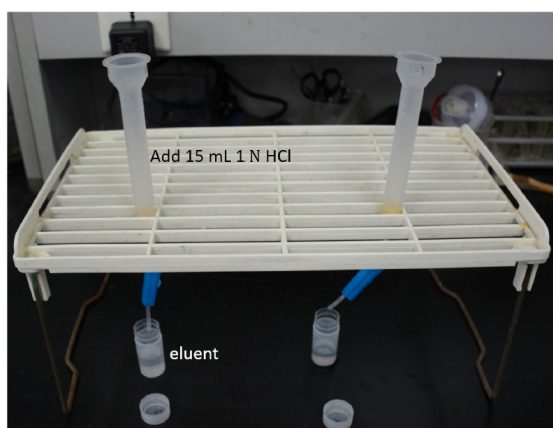


Figure 3. The equipment for eluting the organic acids

4. Then, evaporate the eluent by using a rotary evaporator at 40 °C, a rotation speed of 120 rpm, and a vacuum pressure of 35 hPa (Figure 4).

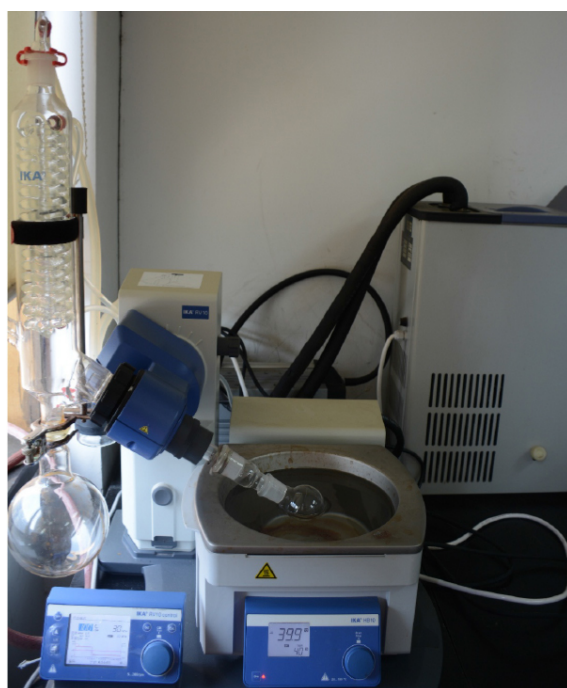


Figure 4. The equipment used in concentrating the organic acids

5. Terminate the evaporation process when the eluent solution has completely dried into powder (approximately 30 min later). Then re-dissolve the powder in 1 ml of distilled water and filter the solution through a 0.2 μ m syringe filter prior to analysis.
6. Detect the organic acid anions by ion chromatography (ICS 3000; Dionex) equipped with an IonPac AS11 anion-exchange analytical column (4 x 250 mm) and a guard column (4 x 50 mm). The mobile phase is 1.2 g/L NaOH at a flow rate of 0.6 ml/min. Generate a standard curve by

using 1, 5, 10, 30, 50, 80, and 100 mg/L of the organic acid (oxalate, malate, and citrate) stock solution.

7. Calculate the organic acid efflux expressing it as a function of the root fresh weight.

Notes:

- a. *The recovery rate of organic acids must be > 80%. To calculate the recovery rate, at least two different concentrations of an organic acid (oxalate, malate, and citrate) stock solution were employed. For example, mix 20 µl or 100 µl 100 mg/L of organic acid stock solution with 1 L of distilled water, and repeat the procedure from 1 to 5 (theoretical concentration in distilled water is 2 or 10 µg/ml). Then, repeat step 6 of procedure and calculate the actual organic acids concentration. Finally, calculate the recovery rate using the following equation: Recovery rate = (Actual value/Theoretical value) x 100.*
- b. *To prepare the anion exchange resin, wash the anion exchange resin powder (500 g) in 1 L 4 N NaOH for about 30 min at room temperature. Repeat three times and wash with distilled water to neutral pH. Wash three more times in 1 L 2 M formic acid for about 30 min each and wash again with distilled water to neutral pH.*
Similarly, wash the cation exchange resin (500 g) four times with 0.5 L 4 N NaOH about 1 h at 60 °C each, then rinse it with distilled water to a neutral pH, afterwards, wash it three times in 0.5 L 2 N HCl about 1 h each at room temperature, then rinse it with distilled water to a neutral pH.
The prepared resins can be stored at 4 °C for at least 1 year.
- c. *This method can be easily adapted to other plant species such as Arabidopsis (Zhu et al., 2012). However, the secretion of organic acids is limited for some small plant species. In this case, the recovery rate can be relatively low, and thus the number of plants per pot must be scaled up in order to collect enough organic acids.*

Data analysis

1. Create a standard curve for each organic acid by plotting the peak area vs. the known concentration.
2. Use the standard curves to calculate the concentrations of each organic acid. This will give the concentration of organic acid present in the eluent. Data can be divided by root fresh weight to determine the amount of organic acids secreted per grams of rice root.

The concentration of organic acids was calculated using the following formula:

$$F = C \times V/W$$

Where,

F is the organic acid secreted in roots (µg/g Fw),

C is the concentration of organic acid (mg/L),

V is the volume of the organic acid (this is 1 ml in the present study),

W is the fresh weigh of 10 rice roots in one pot (g Fw)

Below is an example of a calculation for the concentration of malate. The area of the sample is 0.0612, which was then used in the standard curve equation $y = 0.0084x + 0.0056$ (Figure 5), which calculated that the concentration of malate is 6.6190 mg/L. The volume of malate in present study was 1 ml, whereas the root fresh weight was 0.6126 g. The obtained value was then used in the above mentioned formula to calculate for the concentration in the roots (10.8048 $\mu\text{g/g Fw}$).

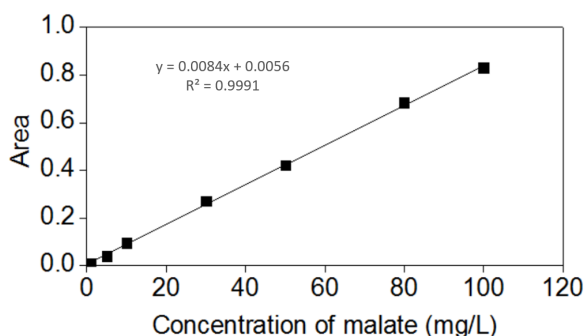


Figure 5. Standard curve of malate

Recipes

1. 1 N HCl

Add ~88 ml 37% HCl to about 500 ml of distilled water, then adjust the final volume of the solution to 1 L using distilled water

2. 2 N HCl

Add ~176 ml 37% HCl to about 500 ml of distilled water, then adjust the final volume of the solution to 1 L using distilled water

3. 2 M formic acid (HCOOH)

Add ~78.6 ml 96% HCOOH to about 500 ml of distilled water, then adjust the final volume of the solution to 1 L using distilled water

4. 4 N NaOH

Add 166.7 g NaOH to distilled water to a final volume of 1 L

5. 0.5 mM CaCl_2 (pH 5.5)

a. 0.5 M CaCl_2 stock solution

Dissolve 73.505 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in distilled water and add distilled water to a final volume of 1 L

b. 0.5 mM CaCl_2

Dilute 5 ml of 0.5 M CaCl_2 stock solution with distilled water to a final volume of 5 L

Adjust the pH of 0.5 mM CaCl_2 solution to 5.5 with 1 N HCl

6. 100 mg/L organic acid stock solution (oxalate, malate and citrate)

100 mg oxalate, malate, and citrate together

Add distilled water to 1 L to make a 100 mg/L stock solution

Dilute the stock solution to 1, 5, 10, 30, 50, 80, and 100 mg/L to generate a standard curve

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