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Rhizosphere Acidification Assay

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[Abstract] Plant survival depends on the ability of root systems to establish themselves in locations where water and nutrients are available for uptake and translocation (Hawes *et al*., 2003). Rhizosphere influences crop productivity by mediating efficient nutrient transformation, acquisition, and use (Shen *et al*., 2013). Rhizosphere acidification is a central mechanism for plant mineral nutrition since it contributes to nutrient solubility and the proton motive force (pmf). This pmf is generated by the plasma membrane H^+-ATPases (Miller and Smith, 1996; Forde, 2000) in root epidermal and cortical cells, and is coupled to active nutrient acquisition (*e.g.,* N, K, P). Roots are able to acidify the rhizosphere by up to two pH units compared to the surrounding bulk soil mainly through the release of protons, but also bicarbonate, organic acids and CO_2. Here we present an easy and inexpensive protocol to quantify protons released to the media by the root system-a method successfully used in our recently published work (Pizzio *et al*., 2015).

Materials and Reagents

1. *Arabidopsis thaliana* seeds (Col-0)
2. Eppendorf tubes (1.5 ml)
3. Commercial Bleach
4. Pipettes and tips (1 and 5 ml)
5. Silicon caps (or aluminum foil)
6. Square Petri dishes
7. Plastic wrap
8. Spatula and forceps
9. Flasks (200 ml)
10. Plastic wrap
11. Glass culture tubes
12. Tween-20 (Sigma-Aldrich, catalog number: P-1379)
13. Murashige and Skoog medium (MS) (*Phyto*Technology Laboratories®, catalog number: M524)
14. Sucrose (VWR International, catalog number: BDH-0308)
15. Potassium Hydroxide (KOH) (Thermo Fisher Scientific, catalog number: P-250)

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16. Agar-agar (Sigma-Aldrich, catalog number: A-1296)
17. Sterile distilled water
18. MES hydrate (Sigma-Aldrich, catalog number: M-8250)
19. Seed sterilization solution (see Recipes)
20. MS solid (see Recipes)
21. MS liquid (see Recipes)
22. Assay solution (see Recipes)

Equipment

1. Autoclave
2. Rotary shaker
3. Flow hood
4. Fridge (4 °C)
5. Plant growth chamber (Conviron, catalog number: ATC26)
6. Magnetic stirrer and stirring bars
7. pH meter (Beckman Coulter)
   Note: pH probe should be capable of measuring pH in samples with volumes <= 3 ml.
8. Balance

Procedure

A. Seed sterilization
   1. Put 5 mg (200-300 seeds) of *Arabidopsis* seeds into an Eppendorf tube and add 750 µl sterilization solution.
   2. Vortex for 15 min at room temperature.
   3. Decant the sterilization solution (with sterile tips) under flow hood and add 750 µl sterile water.
   4. Vortex briefly.
   5. Repeat steps c and d 3 times.
   6. Stratify the seeds in 750 µl sterile water at 4 °C for 2 d in the dark.

B. Plating seeds (under flow hood)
   7. Sow seeds (about 10) on sterile solid MS plates. Keep a density of approximately one seed per cm^2. Crowded plates produce unhealthy seedlings.
   8. Close and seal plates with plastic wrap. Use only one layer of plastic wrap. More than one layer prevents gaseous exchange and healthy plant growth.
   9. Incubate plates in a vertical position in a growth chamber at 21 °C with a 12-h-light/12-h-dark cycle (150 µmol m^{-2} s^{-1}) (Forde, 2000). Vertical growth reduces root stress and prevents agar transfer when transplanting seedlings.

10. Grow seedlings for 5-7 d.

C. Plant growth in liquid media

11. Prepare 200 ml flasks with 4 ml liquid MS each.
12. Close flasks with a “silicon” cap (or with aluminum foil).
13. Autoclave flasks at 121 °C for 20 min.
14. Let flasks cool in flow hood.
15. Transfer 10 seedlings into each flask. Put seedlings in the bottom with the help of sterile spatula and forceps. Be sure that the seedlings stay in contact with the media.
16. Close flasks with a layer of plastic wrap. Transparent plastic wrap favors transmission of light, promotes uniform plant growth, and prevents media evaporation.
17. Grow plants on a rotary shaker at 50 rpm in chamber at 21 °C with a 12-h-light/12-h-dark cycle (150 µmol m^{-2} s^{-1}) for 2 weeks.
18. Pay attention: usually seedlings consume all the liquid media before the 2-week period. In this case, add 4 ml sterile liquid MS.

D. Acidification assay

19. Empty flasks and wash roots with the assay solution (5 ml) for 5 min.
20. Empty flasks again and add fresh assay solution (3 ml).
21. Prepare a control flask with assay solution (3 ml), but without any plants.
22. Close flasks with plastic (transparent) foil and keep them in the growth chamber for 6 h.
23. Transfer all the 3 ml of assay solution from flasks to a glass tube and proceed to pH measurements.
24. Take out plants from flasks and check the number of plants in each flask.
25. Dry plants with paper towels, cut roots from shoots, and immediately weigh to avoid plant dehydration, and the concomitant weight loss. Root and shoot weights are important values because we can either calculate protons released per gram of root or seedling.

E. Calculation

26. Use pH values from each flask to compute $[H^+]$ (mole/L) in sample and control using the equation pH=-log $[H^+]$.
27. Calculate the number of H^+ moles in 3 ml of experimental and control samples [mole/ 3 ml].
28. Compute crude H^+ released to the medium by subtracting the average values of $H^+_{control}$ from H^+_{sample}. A typical experiment requires from 3 to 5 independent replicates.
29. Relativize average number of H^+ moles to either plant number, or to root or plant fresh weight. The units are going to be either mole/plant or mole/g. These relativized values allow us to calculate an average from several flasks and also to compare flasks with different plant numbers or plant lines.

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Representative data

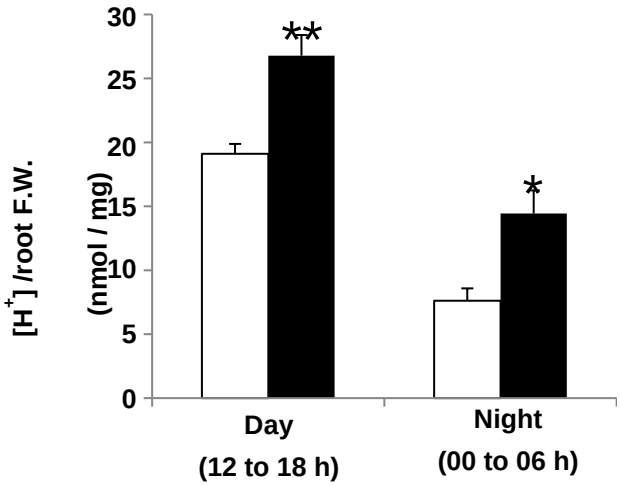

Figure 1. Protons released from the roots during day and night hours by Col-0 (empty bars) and transgenic plants overexpressing the type I H^+-PPase AVP1 (*AVP1-1;* Pizzio *et al*., 2015) (black bars) plants grown in liquid media (mean ± SE; n= 6 pools of 10 plants per line, per time of day, per trial; two independent trials)

Recipes

1. Seed sterilization solution
   30% (v/v) commercial bleach
   0.05% (v/v) Tween-20
2. Murashige and Skoog (MS) solid
   One-half-strength MS
   1% [w/v] Sucrose
   pH 5.7 with KOH
   1% [w/v] agar
3. MS liquid
   One-half-strength MS^+
   1% [w/v] Sucrose (pH 5.7 with KOH)
4. Assay solution
   One-quarter-strength MS
   2 mM MES buffer (pH 6.8 with KOH). Low MES concentration assures similar starting pH for all plants.

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