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Quantification of Sodium Accumulation in *Arabidopsis thaliana* Using Inductively Coupled Plasma Optical Emission Spectrometery (ICP-OES)

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[Abstract] Salt stress is a major issue for plants growing in both natural and agricultural settings (Deinlein *et al.*, 2014). For example, irrigation can lead to the build up of salts in the soil as the irrigation water evaporates, leading to salinization, inhibition of plant growth, reduced productivity and eventually to loss of agriculturally usable land. One key element in trying to understand how salt stress impacts plant growth and development, in defining plant salt sensing and response mechanisms and eventually in the breeding or engineering of plants resistant to this stress is monitoring their salt uptake and redistribution. Methods such as imaging Na-sensitive fluorescent probes (Kader and Lindberg, 2005) and use of Na-ion selective microelectrodes (Shabala *et al.*, 2005) offer the potential to follow Na levels in the plant in a non-destructive manner but are technically demanding and not applicable to field, or even many laboratory, conditions. However, tissue sampling followed by inductively coupled plasma spectroscopy (ICP) represents a simple, quantitative assay to monitor total Na levels in plant samples. ICP analysis is also applicable to plants in any environment where samples can be harvested. The approach uses tissue digestion in acid solutions, followed by injection of the resulting sample into an inductively coupled plasma spectrometer and monitoring the characteristic emitted spectrum from Na. As Na is stable, no complex sample preservation is required. Care needs to be taken with possible Na contamination in standards and samples from the water used for sample preparation and from glassware but otherwise, the approach is simple and robust.

Materials and Reagents

1. Plant tissues
2. 60% (vol/vol) Perchloric acid ($HClO_4$) (Sigma-Aldrich, catalog number: 244252***)***
3. Nitric acid (HNO_3) (Sigma-Aldrich, catalog number: 225711)
4. 18 megaohms (MΩ)-cm deionized water (DI water)
5. NaCl standards, see Recipes for two methods of preparing Na standards
6. ICP-OES calibration standard (Agilent, catalog number: 6610030700)
7. Kimwipe strip, W x H: 30 x 5 mm (manually prepared by cutting a single layer of Kimwipe) (Kimtech Science Kimwipers, catalog number: 34120)
8. NaCl standards (see Recipes)
9. Alternative method of preparing Na standards from 1 M NaCl standard (see Recipes)

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10. Composition of plant growth medium (see Recipes)

Equipment

1. Perkin-Elmer Optima 2000DV inductively coupled plasma optical emission spectrometer (ICP-OES) or similar machine
2. Fume hood
3. Heat block for heating glass test tubes
4. Disposable borosilicate glass tubes with plain ends (O.D. x L: 16 x 125 mm) (Thermo Fisher Scientific, catalog number: 14-961-30)
5. Sterile square Petri dish with grid (L x W x H: 100 mm x 100 x 15 mm, grid of thirty-six 13 x 13 mm squares) (Thermo Fisher Scientific, catalog number: 08-757-11A)

Procedure

This experiment is designed to follow uptake of Na by the root tip and then its transport to the aerial parts of the seedling (Choi *et al.*, 2014).

1. Grow *Arabidopsis* wild type Columbia_0 (WT Col_0) on the surface of plant growth medium [including 0.5% (w/v) Phytagel] in 100 x 100 mm square Petri dishes for 10 days under long day conditions (16 h light/8 h dark).
2. Cover the root tip (~5 mm of the root apex) for 1 h with a single layer Kimwipe strip (W x H: 30 x 5 mm) wetted with 50 µl of DI water (Figure 1A).
3. After this acclimation period, soak the Kimwipe strip for a further 10 min with either 50 µl of DI water (control) or 50 µl of 100 mM NaCl (10 min salt stress).
4. During these control and salt treatments, the Petri dishes should be raised ~15 mm at one end by placing them on a second empty Petri dish. This second Petri dish should be placed under the end of the Petri dish furthest from the seedling roots and closest to the cotyledon/shoot end of the plants (Figure 1B). This protocol ensures that the water or NaCl applied to the Kimwipe strip cannot passively flow along the surface of the plants toward the shoots.

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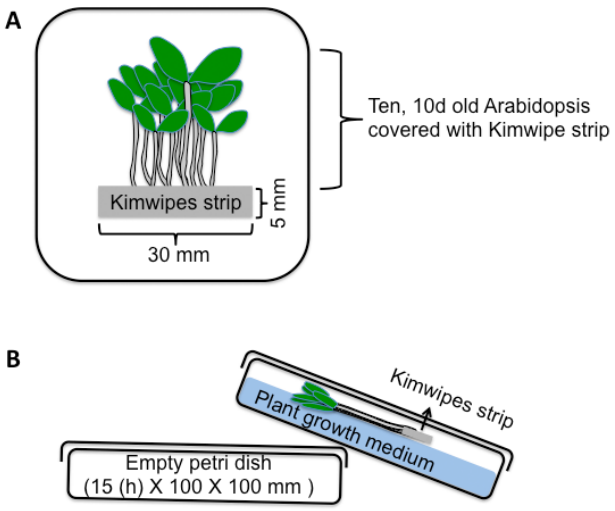

Figure 1. Salt stress experiment performed in a square Petri dish. A. Top view of 100 x 100 mm Petri dish with ten, 10 d old *Arabidopsis* (seedlings). The root tips are covered with a single layer Kimwipe strip (W x H: 30 x 5 mm). B. Side view of Petri dish with raised shoot end to prevent passive liquid flow to shoot tissue. The petri dish is covered with a lid to keep humidity high inside of the Petri dish.

5. Quickly rinse the tissues to be analyzed in DI water to remove any Na passively adhering to their surface.
6. Harvest ~50 mg of fresh tissues and record the weight of each sample. For example, to obtain samples of ~50 mg from roots or shoots you will need at least 10 seedlings of 8-10 day old *Arabidopsis* plants per sample. You will also need to plan for at least three independent experiments with three technical replicates per experiment for robust statistical analyses.
7. In a fume hood, digest each sample in a separate glass test tube (washed with 1% (vol/vol) nitric acid and dried prior to tissue digestion) in 0.6 ml of nitric acid at 120-150 °C for ~2 h or until all the plant tissues are completely dissolved. The 120 °C temperature is readily maintained using a heat block.
8. Add a further 0.4 ml of 60% (vol/vol) $HClO_4$ and incubate at 150-180 °C for an additional 2 h or until the total sample volume is reduced to ≤ 0.5 ml. There is no need to constantly shake or mix the sample.
9. Cool to room temperature and add DI water up to 5 ml.
10. The sodium concentration can now be determined using a Perkin-Elmer Optima 2000DV inductively coupled plasma optical emission spectrometer (ICP-OES) or similar equipment according to the manufacturer's instructions. For example, parameter settings for a Perkin-Elmer Optima 2000DV are 0.60 L/min Nebulizer flow rate, 1 ml/min sample flow rate and 4 ml of total sample usage for 4 replicates (1 ml of each replicate).
11. Raw results of Na level can be converted to parts per million (milligrams per liter) using a standard curve (Figure 2). To make this standard curve, Na standard concentration

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values in ppm (mg/L) were plotted as the x-axis values and corresponding corrected/normalized Na intensity values (Table 2) were then input as the y-axis values. These data can then be converted to milligrams per kilogram fresh weight using the following equation (1):

$$\text{Na (mg/kg fresh weight)} = \frac{\text{(Na level in mg/L) x 1,000] x (Sample volume in L)}}{\text{Sample fresh weight (g)}} \quad \text{(Equation 1)}$$

12. Data from Na standards are shown in Tables 1-2 and Figure 2. Typical data from this experiment are shown in Tables 3-6 and Figure 3:
    a. Table 1, raw Na intensity values obtained from Na standards.
    b. Table 2, corrected/normalized Na intensity values of Na standards using the obtained raw Na standard intensity values in the Table 1. Method for correction/normalization is described in the Table 2 'Note'.
    c. Figure 2, standard curve of corrected/normalized Na intensity values.
    d. Table 3, raw data of Na intensity values of the control and 10 min salt treated *Arabidopsis* samples.
    e. Table 4, corrected/normalized Na intensity values obtained from Table 3.
    f. Table 5, Na levels in ppm (mg/L) of the control and 10 min salt treated *Arabidopsis* seedlings. Conversion of Na intensity values to ppm (mg/L) was done using equation 2, which was obtained based on linear regression analysis of the corrected Na standards (Figure 2).

$$\text{Na (mg/L)} = \frac{\text{(Na intensity values + } 1.706e^{+006})}{5.545e^{+006}} \quad \text{(Equation 2)}$$

    g. Table 6, final conversion of Na levels in Table 5 to 'mg/kg fresh weight (F.W.)' using equation 1 (above step 11).
    h. Figure 3, comparison of Na level in mg/kg F.W. in the roots of 10 old *Arabidopsis* seedlings in response to 100 mM salt treatment.

Representative data

Table 1. Table of raw intensity values from Na standards detected by ICP-OES

Na concentration in standard (µM)	Intensity value				
	Replicate #1	Replicate #2	Replicate #3	Replicate #4	Average
0 (DI water)	-13101	-12852	-13607	-13906	-13367
1	62192	62167	62641	62635	62409
10	207932	206271	203571	207676	206363
100	8783775	8703642	8666576	8517874	8667967
200	20306803	20237705	20096115	20508961	20287396
500	63460855	63572884	64524580	64081778	63910024

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Table 2. Table of corrected* intensity values from raw Na standard values

Na concentration in standard (µM)	Corrected intensity value				
	Replicate #1	Replicate #2	Replicate #3	Replicate #4	Average
0 (DI water)	265	515	-241	-539	0
1	75559	75533	76007	76001	303101
10	221299	219637	216938	221043	878916
100	8797141	8717009	8679942	8531241	34725332
200	20320170	20251071	20109481	20522327	81203049
500	63474222	63586251	64537946	64095145	255693563

*Note: *Na intensity is corrected/normalized by subtracting the average of the raw Na intensity values in the DI water from the raw Na intensity values in each Na standard. For example, in the above dataset, the average Na signal intensity values in DI water = ((-13101) + (-12852) + (-13607) + (-13906))/4 = -13367. Corrected/Normalized intensity value of replicate #1 of DI water is then -13101 - (-13367) = 265.*

The equation obtained from the representative Na standard curve in Figure 1 is $Y = 5.545^{e+006}X - 1.706e^{+006}$ where Y is the corrected Na signal intensity and X is the Na level in mg/L. Therefore, Na level in mg/L from an unknown sample can be calculated using equation 2.

$$X = \frac{(Y + 1.706e^{+006})}{5.545e^{+006}} \quad \text{(Equation 2)}$$

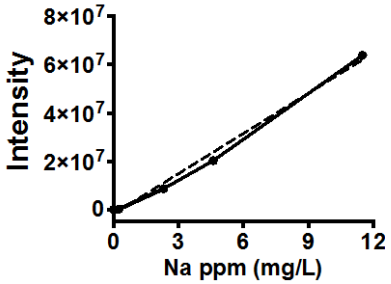

Figure 2. Na standard curve of corrected Na signal intensity. Na standard graph was generated using GraphPad Prism (Ver. 6) software (www.graphpad.com). Solid line represents corrected Na intensity (y axis) to corresponding Na concentration in ppm (mg/L, x axis). Dashed line indicates linear regression analysis of corrected/normalized Na standard (R^2, 0.9912; slope, $5.545e^{+006}$ ± 111579; Y-intercept when X = 0, $-1.706e^{+006}$ ± 573942; *P* value, < 0.0001).

Table 3. Raw data of Na signal intensity values from control and 10 min salt stressed

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***Arabidopsis* seedlings**

	Signal intensity				
	Replicate #1	Replicate #2	Replicate #3	Replicate #4	Average
Control root #1	14268939.3	14515870.1	14259302.6	14529114.1	14393306.5
Control root #2	18390245.8	18113970.6	18376953.2	18586787.6	18366989.3
Control root #3	15961897.1	15946196.2	16018475.1	16181451.1	16027004.9
10 min salt stress root #1	16524276.7	16462936.7	16385847.1	16373113.5	16436543.5
10 min salt stress root #2	16710847.8	16254854.6	16242386.2	16349709.2	16389449.5
10 min salt stress root #3	16676297.9	16596706.1	17026112.0	17105345.0	16851115.3

Note: Na detection using ICP-OES was repeated 4 times (4 technical replicates) from three independent biological replicates (roots #1-#3) for each treatment (for a total of 12 replicates).

Table 4. Corrected/Normalized Na signal intensity values of raw data from Table 3

	Corrected/Normalizes signal intensity				
	Replicate #1	Replicate #2	Replicate #3	Replicate #4	Average
Control root #1	14282305.8	14529236.6	14272669.1	14542480.6	14406673.0
Control root #2	18403612.3	18127337.1	18390319.7	18600154.1	18380355.8
Control root #3	15975263.6	15959562.7	16031841.6	16194817.6	16040371.4
10 min salt stress root #1	16537643.2	16476303.2	16399213.6	16386480.0	16449910.0
10 min salt stress root #2	16724214.3	16268221.1	16255752.7	16363075.7	16402816.0
10 min salt stress root #3	16689664.4	16610072.6	17039478.5	17118711.5	16864481.8

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Table 5. Conversion of Na signal intensity values to concentration in ppm (mg/L)

	Na level (mg/L)					
	replicate #1	replicate #2	replicate #3	replicate #4	Average	Standard deviation (SD)
Control root #1	2.883	2.928	2.882	2.930	2.91	0.027
Control root #2	3.627	3.577	3.624	3.662	3.62	0.035
Control root #3	3.189	3.186	3.199	3.228	3.20	0.019
10 min salt stress root #1	3.290	3.279	3.265	3.263	3.27	0.013
10 min salt stress root #2	3.324	3.242	3.239	3.259	3.27	0.040
10 min salt stress root #3	3.318	3.303	3.381	3.395	3.35	0.045

Note: Na intensity from the samples can be converted into ppm (mg/L) using the equation 2.

Table 6. Conversion of Na level to 'mg/kg fresh weight (F.W.)

	Na level (mg/kg F.W.)					
	replicate #1	replicate #2	replicate #3	replicate #4	Average	Standard deviation (SD)
Control root #1	2151.8	2185.0	2150.5	2186.8	2168.51	20.096
Control root #2	2706.4	2669.3	2704.6	2732.9	2703.30	26.116
Control root #3	2379.6	2377.5	2387.2	2409.2	2388.38	14.473
10 min salt stress root #1	2611.2	2602.4	2591.4	2589.6	2598.64	10.116
10 min salt stress root #2	2518.0	2455.7	2454.0	2468.7	2474.08	29.997
10 min salt stress root #3	2047.9	2039.0	2086.8	2095.6	2067.31	28.055

Note: Na level in ppm (mg/L) in Table 5 was converted to Na level in 'mg/L fresh weight' using the equation 1.

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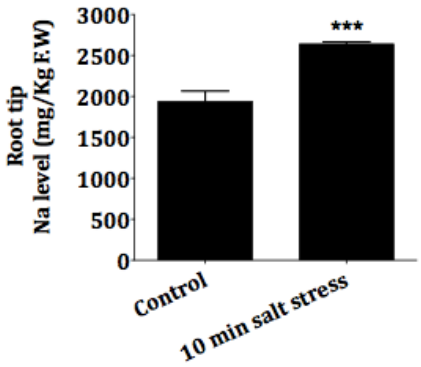

Figure 3. Comparison of Na level (mg/kg F.W.) in the roots of 10d old *Arabidopsis* in response to salt stress. Root tips of 10 d old *Arabidopsis* seedlings were subjected to salt stress with 100 mM NaCl for 10 min as described above. Error bar, standard deviation of n = 12 ($P < 0.001$). ***Student's t-test p value < 0.001 compared to Control.

Notes

1. Some sources of water contain high levels of Na that will provide a background to each sample. It is important to run a calibration on the ICP machine being used with an ICP-OES calibration standard according to the manufacturer's instructions. One commercial calibration mix for ICP-OES from Agilent Technologies, contains 500 mg/L Ca, Fe, K, Mg and Na in 5% (v/v) HNO_3. Once the machine is calibrated, measure a Na calibration curve (see Recipes), paying special attention to the 0 Na point to establish if significant Na contamination is occurring. Using DI water is essential but contamination may also occur if the equipment has been previously used for samples with high Na content. Thorough flushing of the equipment with DI water is needed under these circumstances.
2. The provided exemplary datasets in this protocol were obtained from 10 day old *Arabidopsis* wild type Columbia-0 ecotype seedlings grown under long day light cycles (16 h light/8 h dark) at 22 °C. Therefore, Na accumulation values might be variable depending on plant species, growth conditions, age, and genotypes.

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Recipes

1. NaCl standards

Na (µM)	NaCl (g/L)
0	0
1	0.000023
10	0.00023
100	0.0023
200	0.0046
500	0.0115

Note: Dissolve NaCl in DI water as indicated.

2. Alternative method of preparing Na standards from 1 M NaCl standard
   a. Prepare 1 ml of 1 M Na standard solution by dissolving 0.023 g of NaCl into 1 ml DI water and vortex until NaCl is completely dissolved.
   b. Dilute 1 M NaCl standard as below.

NaCl standard	Volume of NaCl standard	Volume of DI water	Dilution factor	Total Volume (ml)
10 mM	10 µl of 1 M NaCl	990 µl	1:100	1 ml
1 mM	1 ml of 10 mM NaCl	9 ml	1:10	10 ml
500 µM	5 ml of 1 mM NaCl	5 ml	1:1	10 ml
200 µM	2 ml of 1 mM NaCl	8 ml	1:5	10 ml
100 µM	1 ml of 1 mM NaCl	9 ml	1:10	10 ml
10 µM	0.1 ml of 1mM NaCl	9.9 ml	1:100	10 ml
1 µM	0.1 ml of 100 µM NaCl	9.9ml	1:100	10 ml

Note: Make sure to make a minimum 5 ml of NaCl standard solutions as one measurement uses 1 ml of each standard solution but replicates for each point are needed.

3. Composition of plant growth medium

   The plant growth medium is half strength Epstein medium consisting of 3 mM KNO_3, 2 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.5 mM $MgSO_4{\cdot}7H_2O$, 1 mM $(NH_4)H_2PO_4$, 0.56 mM *myo*-inositol, 2.3 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mM sucrose, micro-nutrients, and 0.5% (w/v) Phytagel at pH 5.7.

   The micro-nutrients are 25 µM KCl, 17.5 µM H_3BO_3, 1 µM $MnSO_4{\cdot}H_2O$, 1 µM $ZnSO_4{\cdot}7H_2O$, 0.25 µM $CuSO_4{\cdot}5H_2O$, 0.25 µM $(NH_4)_6MoO_{24}{\cdot}4H_2O$, and 25 µM (ethylene-dinitrilo)tetraacetic acid (Fe-Na EDTA).

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