FROM: Sunkar, Ramanjulu (Ed) 2010. Plant Stress Tolerance. Methods and Protocols. Springer.

Chapter 23

Measuring Soluble Ion Concentrations (Na^+, K^+, Cl^-) in Salt-Treated Plants

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Abstract

The control of Na^+ and Cl^- uptake from soils, and the partitioning of these ions within plants, is an essential component of salinity tolerance. Genetic variation in the ability of roots to exclude Na^+ and Cl^- from the transpiration stream flowing to the shoot has been associated with salinity tolerance in many species. The maintenance of a high uptake of K^+ is also essential, so measurements of Na^+, K^+ or Cl^- are frequently used to screen for genetic variation in salinity tolerance. As these ions are not bound covalently to compounds in cells, they can be readily extracted with dilute acid. Na^+ and K^+ can be measured in a dilute nitric acid extract using a flame photometer, by atomic absorption spectrometry or by inductively coupled plasma (ICP)–atomic emission spectrometry. Cl^- can be measured in the same acid extract with a chloridometer or colorimetrically using a spectrophotometer.

Key words: Sodium, potassium, chloride, ion extraction, ion analyses, salinity.

1. Introduction

The control of Na^+ and Cl^- transport is a critical requirement for salinity tolerance in plants. If excessive amounts of Na^+ or Cl^- enter plants, these ions typically rise to toxic levels in the older transpiring leaves. Because leaves evaporate about 50 times more water than they retain, Na^+ and Cl^- will quickly reach high concentrations there, unless the roots can restrict the movement of these ions into the transpiration stream. Salt-tolerant plants should therefore exclude most of the NaCl in the soil solution, ideally 98% (1). On the other hand, accumulation of a certain amount of Na^+ or Cl^- in leaves is essential for osmotic

R. Sunkar (ed.), *Plant Stress Tolerance*, Methods in Molecular Biology 639,
DOI 10.1007/978-1-60761-702-0_23,

adjustment. The partitioning of Na^+ and Cl^- into the vacuole, accompanied by the accumulation of K^+ or organic solutes in the cytoplasm, is an important mechanism of salinity tolerance for halophytes (2). Genetic variation in the accumulation of Na^+ and Cl^- is associated with salinity tolerance in many species. The maintenance of a high K^+/Na^+ ratio in the cytoplasm is also essential (3).

Some of the genes controlling Na^+ uptake and transport have been cloned (3), but much more remains to be learnt about the control of Na^+ and Cl^- concentrations in various compartments and transport of these ions at the intracellular, cellular, organ and long-distance transport levels. There is therefore a frequent need to measure the concentrations of Na^+, K^+ and Cl^- in various plant organs and tissues, both in the search for natural variation and the analysis of transgenic lines. Considerations of experimental design, of the time frame measurements should be taken, and the critical tissues to be sampled, are given in the accompanying chapter in this volume (4).

At any one time, the concentration of Na^+ will vary from leaf to leaf and between locations within a single leaf. The concentration is always less in younger than older leaves (5). As ion concentrations continually change with time in individual leaves (5, 6), it is important, when comparing differences in Na^+ uptake between different genotypes, to take the leaves at the same stage of development or at the same period of exposure to the NaCl (7). A leaf blade at a defined stage of development is the recommended tissue to sample, for example in wheat, leaf 3 at 10 days after emergence (7). The concentrations may be quite different in different leaf parts: between base and tip of a leaf blade (8), between blade and petiole (9) or between blade and sheath (6, 8). These differences are not fixed in time. The gradients in Na^+ and K^+ along a leaf change with age of the leaf both in degree and in direction (8).

In plant cells, Na^+, K^+ and Cl^- are largely present in soluble form and can be recovered with a dilute (0.5 M) acid extract (10). Protons displace the ionic bonds between Na^+ and K^+ to the negative charges on proteins and other macromolecules, and if nitric acid (HNO_3) rather than hydrochloric acid (HCl) is used, the NO_3^- replaces the ionic bonds between Cl^- and macromolecules. A conventional acid digest, using concentrated nitric acid at about 150°C, which completely solubilizes the plant tissue, is unnecessary to extract these three ions as they are not bound to cell constituents by covalent bonds.

To measure Na^+ and K^+, three atomic spectroscopy techniques are currently in use in various laboratories: flame photometry, atomic absorption spectrometry and inductively coupled plasma–atomic emission spectrometry (known as ICP). The latter is often the preferred method as it can measure a number of

elements simultaneously and over a very wide concentration range but is more expensive and confined to well-equipped analytical laboratories.

All three instruments operate under a similar principle. Solutions are aspirated into the excitation region where high-temperature atomization sources (flame or plasma) provide energy, which excites the electrons in atoms to higher energy levels. Energy is released by the electron as it returns to its original state. These energies are detected as light at a particular wavelength. The fundamental characteristic of this process is that each element absorbs and emits energy at specific wavelengths peculiar to its chemical composition. The intensity of the energy emitted at the chosen wavelength is proportional to the concentration of that element in the aspirated solution. By determining which wavelengths are absorbed or emitted by a sample and by measuring their intensities in relation to a reference standard, the concentrations of elements in the sample can be calculated.

Flame photometry is an atomic emission method for the routine detection of Na^+ and K^+. It uses a readily available hydrocarbon gas such as propane. Optical filters select the emission wavelength for the element. A calibration curve is made with solutions of known concentration, and comparison of emission intensities of samples to those of standard solutions allows quantitative analysis of the element of interest in the solution.

Atomic absorption spectrometry is a more sensitive analytical method. It uses the absorption of light to measure the concentration of specific atoms. A hollow cathode lamp of the element being measured is used as the light source. A mixture of air and acetylene produces a high-temperature flame into which samples are introduced. The atoms in the sample absorb light from the source and the electrons move to higher energy levels. A light detector set at a particular wavelength (monochrometer) measures the light absorbed by that element.

An inductively coupled plasma (ICP) is a very high-temperature excitation source (7,000 K) that ionizes atoms. Coupled with a high-resolution atomic emission spectrometer, it is used for quantitative, simultaneous measurement of a large range of elements. Samples are aspirated into the plasma gas (argon). The high temperature provides sufficient energy to enable the excited atoms to move to higher energy levels. When the atoms decay, they emit characteristic light, which is detected simultaneously using polychromators and multiple detectors or camera chips.

To measure Cl^-, the most common instrument is a chloridometer, which titrates the Cl^- with Ag^+ released from a silver wire. The principles of operation of the chloridometer are that Ag^+ is released at a constant rate as current is passed between a

pair of silver generator electrodes. Cl^- in the sample solution precipitates with the generated Ag^+ as AgCl. After all the Cl^- in the sample have precipitated, there is a steady increase in Ag^+ in solution that produces an increasing current through a pair of silver indicator electrodes. In the presence of an increase in indicator current, a relay will be activated, and a timer, which runs concurrently with Ag^+ generation, is automatically stopped (amperometric indication). As the rate of generation of Ag^+ is constant, the amount of Cl^- precipitated from the sample is proportional to the elapsed time. Thus, solutions with higher amounts of Cl^- take a longer time to reach the endpoint, and Cl^- is quantified by making a standard curve to relate time to known concentrations of Cl^- in standard solutions.

If a chloridometer is not available, Cl^- can be measured by a colorimetric "ferricyanide" method. Thiocyanate ion is liberated from mercuric thiocyanate by the formation of soluble mercuric chloride. In the presence of ferric ion, free thiocyanate ion forms a highly coloured ferric thiocyanate (11).

It is possible to measure Cl^- with a "Cl^--specific electrode", but details are not presented here.

The reliability of these techniques, like all analytical methods, relies on the use of appropriate standards, blanks, reference samples and spikes. A blank is an assay unit taken through all the same procedures, but lacking any plant tissue, so as to check for any background contamination. A reference sample is a plant tissue, verified to contain a known nutrient concentration, taken through the procedure with each batch of samples so as to determine reliability of each set of analyses over time. A spike is the addition of a known amount of the compound being analysed to a selected group of tissue extracts so that percent recovery can be determined.

2. Materials

2.1. Laboratory Items

1. HNO_3 A.R. grade.
2. NaCl A.R. grade.
3. KCl A.R. grade.
4. Distilled or de-ionized water.
5. Volumetric glassware (1 L and 100 mL).
6. Measuring cylinder (50 mL), pipettes.
7. Analytical balance.

8. Plastic tubes (10 mL) with screw tops.
9. Oven set at 60–70°C or shaker at room temperature.

All glassware should be washed in dilute nitric acid so that it is free of ions.

2.2. Sample Preparation

The dilute acid extraction based on Hunt (10) can be varied in detail depending on the amount of tissue and whether it is thin or soft and therefore readily permeated by the acid, or whether it is bulky or fibrous and should be ground first.

2.2.1. Leaf Blades

The surface of the leaves should be rinsed in distilled water if there is concern that the surface might be contaminated by handling or by splashing of saline solution. If the concentration of ions on a fresh weight or a water basis is required, the leaves should be weighed fresh by handling with forceps or rubber gloves and then placed in small envelopes or vials and dried in an oven at 60°C–70°C for 2 days. Individual leaf blades can be extracted in whole and the extract sampled directly for analysis (*see* **Notes 1** and **2**).

2.2.2. Roots

The surface of roots should be rinsed in solution, but quickly as there will be rapid efflux of Na^+ from the apoplast and epidermal cells. Two 10-s dips in 10 mM $CaNO_3$ is recommended. If the roots have been in high concentrations of salt (over 150 mM NaCl), it is advisable to rinse them in "iso-osmotic solutions" so as to avoid turgor loss due to osmotic shock. Organic osmotica such as sorbitol, mannitol, or high molecular weight PEG have been used by various laboratories to make iso-osmotic solutions. $CaNO_3$ should again be present. It is difficult to get a true fresh weight of roots without adhering solution on the one hand or damage due to heavy blotting on the other hand. Roots are then placed in small envelopes or vials and dried in an oven at 60°C–70°C for 2 days. Individual roots can also be extracted in whole and the extract sampled directly for analysis (*see* **Notes 1** and **2**).

Whole shoots or leaves of woody perennials need to be chopped into small pieces or ground, and the extract should be filtered or centrifuged before an aliquot is taken for analysis.

2.3. Grinding

Large or woody samples should be ground to a fine powder to achieve a homogenous representative sample for ion analysis.

1. Vials and metal spatula for collecting sample from grinder.
2. Dust mask.
3. Ear plugs.
4. Safety glasses.
5. Air-tight bags with desiccant for storing samples.

3. Methods

3.1. Dilute Acid Extraction

1. Make up 1 L of 0.5 M nitric acid with HNO_3 and distilled or de-ionized (e.g. Milli-Q) water. In a fume cupboard, add about 900 mL of distilled water to a 1-L volumetric flask, then 31.9 mL of concentrated HNO_3 using a graduated pipette to which a hand-held suction device is fitted, or a 50-mL measuring cylinder. Do not use your mouth to suck the concentrated acid into the pipette. Mix by stopping the flask and inverting several times, slowly. Add sufficient distilled water to make up to the 1 L mark. Always add the acid to water, as mixing the two is an exothermic reaction and it can "spit" if mixed the other way round.
2. Weigh out approximately 100 mg of dried and ground plant tissue into a tared 10-mL plastic tube and record the exact weight. Alternatively, place a pre-weighed leaf into the 10-mL tube. Add 10 mL of 0.5 M HNO_3.
3. For smaller samples of plant tissue, extraction volumes should be reduced: For 20–50 mg, use 5 mL and for <20 mg, use 2.5 mL. For samples larger than 100 mg, use 50-mL plastic tubes and increase the volume of dilute acid accordingly, i.e. for 200 mg, use 20 mL, etc.
4. Include "blanks" consisting of only the dilute acid and no tissue at the start and end of each set, as well as any time a new source of dilute acid or vials is used.

   Include reference material with known ion concentrations and prepare as per experimental samples. A number of organizations – NIST (USA), IAEA, BCR (Europe) and ASPAC (Australia) – supply dried plant tissue with certified concentrations of Na^+, K^+ and Cl^-.
5. Tightly screw on the lids for all samples and shake well to suspend all plant materials in the dilute acid. Place all vials tightly in a box and place on a shaker for 2 days at room temperature. Alternatively place in an oven at 80°C for 1 h. Mix by inverting tube once during that time and once after cooling.
6. After extraction, allow solid material to settle to the bottom of the vial. For dilutions, pipette from the middle of the extract, being careful not to take any solid or suspended material. Samples should be filtered or centrifuged if necessary.
7. Dilute extracts using only distilled or Milli-Q water.

3.2. Flame Photometer

3.2.1. Standards

For Na and K: Make up a stock of 50 μg/mL K^+(e.g. use KCl) and Na^+(e.g. use NaCl) and dilute accordingly to make up 0, 0.5, 1, 2.5, 5, 10, 15, 20 and 25 μg/mL standards of Na^+ and K^+. Ensure that the salts used are dry (e.g. oven dry at 60°C). Alternatively, standards can be purchased from commercial suppliers.

3.2.2. Dilutions

The dilution needed for the extracts will vary depending on the salt concentration of the growth medium, the tissue being analysed and the plant species being studied. The dilutions are required to provide samples with K^+ and Na^+ within the range of the standard curve. The following is an approximate guide for leaf tissue from a non-halophyte grown in 100 mM NaCl. We recommend always doing "trial" dilutions for each treatment, tissue and species before diluting all extracts.

1. K^+: Dilute controls 1:100 in a total of 10 mL, e.g. 100 μL of extract plus 9,900 μL of Milli-Q water. Dilute saline samples 1:50.
2. Na^+: Controls will often not need diluting but then should be run with standard solutions in the same matrix (i.e. 0.5 M nitric acid), as the standard curve shifts. Dilutions of 1:5 would typically enable standards in water to be used. Dilute saline samples 1:10 to 1:50, depending on which tissue is being assayed (e.g. Na^+ is higher in older leaves), in a total volume of at least 2 mL.

The following instructions were developed based on experience with Corning (model 410) and Jenway (model PFP7) flame photometers, but the overall approach should, with some modifications, be applicable to other instruments.

3.2.3. Operation

1. Ensure that the U tube is full of water (check by giving a squeeze) and make sure that the nebulizing tube is in a beaker full of de-ionized (DI) water. (Make sure that this tube is always in DI water between samples.)
2. Turn on air, gas, power switch. Hold the ignition switch until the flame is strong.
3. Set to Na or K.
4. Allow 30 min for the machine to warm up and stabilize.
5. Aspirate the most concentrated standard first (10 μg/mL K^+, 25 μg/mL Na^+) and adjust the coarse and fine tuning dials to display ~150 for Na^+ and ~100 for K^+.
6. Aspirate the 0 μg/mL standard and use the blank dial to adjust to 0. Aspirate all standards and obtain a linear standard curve.

7. Aspirate samples until a steady reading is obtained. Approximately every 10 samples repeat one of the standards so as to check that the machine has not drifted. Redo standard curve when drift has occurred (or about every hour).
8. When finished, leave tube in DI water for about 10 min, then turn off switch, gas, then air, followed by the power.

3.2.4. Calculating Concentrations

1. Convert the readings from the flame photometer to micrograms per millilitre using the standard curve.
2. Convert micrograms per millilitre into micromole per gram DW = (concentration in micrograms per millilitre × dilution factor, e.g. 100 for 1:100 dilution × volume of dilute acid extract, e.g. 10 mL) / (DW of tissue used in extraction × MW of ion 39.098 for K^+ and 22.99 for Na^+).

3.3. Atomic Absorption Spectrometry

1. Remove about 3 mL of solution from each tube and place in clean 10-mL tubes and give to operator with blanks.
2. Construct a spreadsheet with the weight of sample and the expected approximate concentration for each sample range as dilutions will need to be done. The operator needs to know whether the samples are controls (in which case a 1:10 dilution may be done) or salt-treated plants (in which case a 1:100 or 1:500 dilution will need to be done). The operator has standards in the range of 0–2 μg/mL. Tell the operator that you need K^+ and Na^+ as two separate measurements need to be made.
3. Calculate as above (step 2 of **Section 3.2.4**).

3.4. ICP

1. Remove about 3 mL of solution from each tube and place in clean 10-mL tubes and give to the operator with blanks. The operator has standards in the range of 0–1,000 μg/mL, which means that dilutions are not needed. Tell the operator which ions you need (*see* **Note 3**).
2. Calculate as above (step 2 of **Section 3.2.4**).

3.5. Chloridometer

3.5.1. Reagents

1. Nitric/acetic acid reagent (0.4 M HNO_3 and 40% acetic acid): Add 25.6 mL concentrated nitric acid to about 500 mL Milli-Q water in a 1-L volumetric flask. Add 400 mL glacial acetic acid slowly to the flask, stirring as you add, and then make up to 1 L. The final concentration in the titration vials should be 0.1 M nitric acid and 10% acetic acid.
2. Gelatin reagent: Dissolve 0.62 g dry gelatin in 100 mL of boiling Milli-Q water in a beaker. Dispense into 25-mL containers and store in fridge for up to 6 months. Bring back to room temperature before using.

3.5.2. Standards

Make up 0, 0.1, 0.3, 0.5, 0.7 and 1 mM Cl^- standards (e.g. use NaCl).

3.5.3. Dilutions

Add volumes of extracts required to give sample dilutions so that Cl^- falls in the range of the standard curve. Examples of dilutions for K^+ and Na^+ are given above.

3.5.4. Operation

The following protocol is for older machines (e.g. Buchler–Cotlove Chloridometer 662201). For newer models (e.g. Sherwood 926), the reagents and principles are the same, except a smaller volume of extract (e.g. 20 μL or 100 μL) is added directly to a 100 mL solution containing the reagents, which needs replacing only every 20 samples.

1. Clean all electrodes thoroughly with silver polish, and rinse, before turning on the machine.
2. Set the range of the machine to medium (0–0.5 mM) unless low concentrations are expected (i.e. less than 0.1 mM), then use the low range.
3. Switch the machine on and after 10 s stop the stirrer by manual shut off. Set titration switch to titrate, move adjustable red pointer to coincide with the indicator black pointer. A click will be heard as the relay is activated. Move the red pointer to about 20 μA (black one will follow).
4. Allow 30 min for machine to warm up.
5. Prepare sample vials: Add 3 mL of diluted extract (or blank or standard), 1 mL nitric/acetic acid reagent and 200 μL of gelatin reagent to glass vials.
6. Condition the electrodes by titration of three blanks (3 mL of blank plus reagents as above). Place the vial into the metal holder and raise so that solution covers the electrodes and the vial fits over the plastic housing above the electrodes. Turn titration switch to adjust. Wait for the black needle to fall to a stable value of <5 μA (will probably take until the third blank). When it does fall below 5, move the red pointer to 10 μA above the black pointer (i.e. about 15). Ensure before each sample that there is a difference of about 10 μA between the red and black needles. Turn the titration switch to titrate. Record the time when the timer shuts off and reset this between samples.
7. Run all the standards to generate a standard curve of time vs millimolar of Cl^-.
8. Run all samples, blanks and reference tests.
9. If the machine is left for >5 min, then another blank must be measured to make sure that the black needle is falling below 5 μA. Run a check standard approximately every 20

samples to check for any drift. If there is drift, it is likely that the electrodes need cleaning with silver polish. Turn off the instrument prior to cleaning electrodes.

10. To re-use glass vials, rinse with DI water and dry with paper towels (*see* **Note 4**).

3.5.5. Calculating Concentrations

1. Convert the readings in seconds to Cl^- concentrations in millimolar using the standard curve.
2. Convert millimolar Cl^- in samples into micromolar per gram DW = (concentration in micromol × dilution factor, e.g. 100 for 1:100 dilution × volume of acid extract, e.g. 10 mL) / (DW of tissue extracted).

3.6. Colorimetric Determination of Cl (Ferricyanide Method)

Ferric thiocyanate absorbs strongly at 480 nm. The calibration fits a second-order polynomial (12).

3.6.1. Reagents

1. Stock mercuric thiocyanate solution: Dissolve 4.17 g $Hg(SCN)_2$ in about 500 mL methanol, dilute to 1,000 mL with methanol, mix and filter through filter paper. *Caution*: Mercuric thiocyanate is toxic. Wear gloves!
2. Stock ferric nitrate solution: Dissolve 202 g $Fe(NO_3)_3 \bullet 9H_2O$ in about 500 mL distilled water, then carefully add 25 mL concentrated HNO_3. Dilute to 1,000 mL with distilled water and mix. Filter through paper and store in an amber bottle.
3. Combined colour reagent: Add 150 mL stock $Hg(SCN)_2$ solution to 150 mL stock $Fe(NO_3)_3$ solution. Mix and dilute to 1,000 mL with distilled water.
4. Stock chloride solution: Dissolve 1.6482 g NaCl, dried overnight at 140°C, in distilled water and dilute to 1,000 mL; 1.00 mL solution is equivalent to 1.00 mg Cl.

3.6.2. Standard Chloride Solutions

1. Prepare a set of chloride standards in the desired concentration range, such as 1, 2, 5, 10, 20, 40, 60, 80, 100, 140, 200 mg/L, using stock chloride solution and making to volume with 0.5 M HNO_3 extractant. N.B. Remember to include some acid blanks.
2. To make up this standard set using 100-mL volumetric flasks, pipette 100 μL, 200 μL, 0.5 mL, 1.0 mL, 2 mL, 6 mL, 8 mL, 10 mL, 14 mL and 20 mL of the standard chloride solution and make up to the mark with 0.5 M HNO_3.

3.6.3. Method

1. Pipette 1 mL of the standard chloride solutions, the samples and blanks into labelled plastic tubes. Pipette 3 mL of com-

bined colour reagent into each tube and mix. The absorption of the resulting colour is measured using a spectrophotometer with the wavelength set at 480 nm.

2. Prepare a standard calibration curve by plotting the measured absorption against the chloride concentration in the standards. Use this curve to determine the chloride concentration of the samples.
3. Report only those values that fall between the lowest and the highest calibration standards. Samples exceeding the highest standard should be diluted and reanalysed.
4. If the supernatant of extracted samples is highly coloured, they may be clarified, before adding the colour reagent, by adding some washed activated carbon, shaking, then filtering. Ensure that a couple of blanks are similarly treated and measured.
5. Report results in milligrams of Cl per litre (*see* **Note 4**).
6. Convert to millimolar Cl and then into micromolar per gram DW (see 3.5.5).

4. Notes

1. A decision must be made to present ion concentrations on a water basis (mM) or dry weight basis (μmol/g DW or mmol/g DW). The water content (amount of water per gram dry weight) is quite different for different parts of a leaf. In monocotyledonous species, the sheath has higher water content than does the blade; in dicotyledonous species, the blade usually has a high water content than does the petiole. Calculation on a water content or a fresh weight basis is therefore not valid for a whole shoot, so ions should be expressed on a dry weight basis.
2. Some laboratories have measured ion concentrations in expressed sap from leaves (13), but as critical toxicity values have been determined on a dry weight basis (14), and as sap ion concentrations can change with changes in water content as well as ion content, this method has not been widely adopted. The method is valid for ion ratios, such as K^+/Na^+, but caution is needed if comparing different treatments as leaf water content can change markedly with different salinity treatments.
3. A wide range of elements in plant tissue can be analysed with ICP-AES. However, this simple dilute acid extraction is not recommended when a complete elemental analysis is required. It has been found that there is incomplete recovery

for some elements, particularly those elements which play a structural role in the plant or are present in a relatively insoluble form. Performing a complete, conventional elemental analysis requires the plant tissue to be digested in concentrated nitric acid or a mixture of nitric/perchloric acids at high temperature (15).

4. It is important to ensure that hazardous chemical waste is disposed of in a manner that complies with local regulations.

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