Continuous Measurement of Macronutrient Ions in the Transpiration Stream of Intact Plants Using the Meadow Spittlebug Coupled with Ion Chromatography

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A method is described for continuous, nondestructive analysis of xylem-borne mineral nutrients in intact transpiring plants. The method uses the xylem-feeding insect the meadow spittlebug (*Philaenus spumarius* L. [Homoptera: Cercopidae]). This insect will feed from a wide range of plant species and organs. Insect excreta can be collected at all times of the day and night, and its mineral ion content can be analyzed rapidly, and without purification, by ion chromatography. The excreta will have a mineral content virtually identical to that of xylem sap. Cages suitable for containing the insects and collecting excreta from any desired location on plants in both laboratory and greenhouse are described. Even in the greenhouse, evaporation had only a minor effect on the sample ion content. Example results are presented which illustrate dynamics, over several days, in the xylem concentrations of sodium (Na$^{+}$), potassium (K$^{+}$), NH$_4$$^+$, magnesium (Mg$^{2+}$), calcium (Ca$^{2+}$), chloride (Cl$^-$), NO$_3^-$, PO$_4^{3-}$, and SO$_4^{2-}$. These data were collected from young plants growing in pots of compost in the laboratory and from fully mature pepper (*Capsicum annuum* L. cv Bellboy) plants growing in hydroponics (rockwool) in the greenhouse. This method should facilitate studies of macronutrient uptake and transport in a range of plants and environments.

Xylem is a defining feature of the higher plant. Its primary function is water transport, but it also plays major roles in mechanical support, in nutrient transport, and in signaling and integration of plant responses (Malone, 1996). Some mineral nutrients, particularly Ca$^{2+}$, move almost exclusively in the xylem (Raven, 1983), whereas others, such as K$^+$ and Mg$^{2+}$, can move via both xylem and phloem.

Despite its importance for crop growth, xylem transport has rarely, if ever, been analyzed directly. This is because of the difficulty of extracting xylem sap without contamination from neighboring tissues and from cells at cut surfaces. It is especially difficult to obtain xylem sap from plants that are transpiring, because in these, the sap is held under strong negative pressure. Penetration by a sampling device is liable to cause rapid cavitation of the xylem. Probes can sometimes be located within the vessels of transpiring plants (Tyree, 1997), but extraction of sap with such probes is not possible when the sap is at strong negative pressure.

Studies of xylem transport have therefore almost invariably used indirect methods to obtain xylem sap: Root exudates can be collected from the cut stumps of detopped plants; xylem sap can be sucked from pieces of excised stem using modest vacuum (Bollard, 1960); sap can be expressed from the cut ends of excised organs using the pressure bomb. These methods have provided a wealth of comparative information on mineral transport, but they are destructive and cannot be used for analysis of nutrient dynamics within the shoot. In addition, the solute composition of fluids extracted by these methods may not match that of the transpiration stream. This is because of contamination from cut cells and other factors. In addition, methods such as root exudation generate fluid by forces and pathways that may differ from those in operation during transpiration, and they may therefore generate sap with a different solute profile.

A promising and mostly noninvasive method for collecting xylem sap was introduced by Gollan et al. (1992). This uses compressed air to drive water up the plant from root to shoot and to force sap expression at a cut petiole. This method requires a pressurized root volume, and it is not applicable to plants in the field. In addition, it cannot be used on plants in hydroponic culture because the roots of such plants become flooded when pressurized. Thus, there remains a need for a method for routine extraction of xylem fluid from intact, transpiring plants. Any such method should ideally be nondestructive and applicable to a wide range of plant species, tissues, and nutrient ions.

Certain insects are believed to feed on xylem sap. A number of workers have suggested that such insects offer insights into the chemistry, physiology, and transport properties of xylem (Mittler, 1967; Newby, 1980; Horsfield, 1978; Andersen et al., 1989, 1992;
Crews et al., 1998). The xylem feeder Homalodisca coagulata has been used to examine C and N dynamics in several plant-insect systems (Brodbeck et al., 1999). Andersen et al. (1989) used a combination of five analytical approaches to determine the mineral nutrient content of H. coagulata excreta.

The meadow spittlebug (Philaenus spumarius) is a common UK insect that feeds on xylem sap. Its prolonged and copious production of watery excreta is compatible only with feeding from the main transpiration stream (Malone et al., 1999). This insect has been used to study Na+ exclusion from the shoot xylem of certain wheat (Triticum aestivum) varieties (Watson et al., 2001) and to investigate insect amino acid preferences (Horsfield, 1978). Here, the meadow spittlebug is used in combination with ion chromatography (IC) for rapid nondestructive analysis of all the macronutrient ions in the xylem of intact, transpiring plants. Example results from plants in laboratory and greenhouse are presented.

RESULTS AND DISCUSSION

IC Assay Performance

IC was used to determine all of the macronutrient cations (Na+, K+, NH4+, Mg2+, and Ca2+) and anions (Cl−, NO3−, PO43−, SO42−) in meadow spittlebug excreta. Performance of the assay was tested by dilution analysis. Dilutions of samples gave results that were linear and parallel to the standard curves for all the ions tested (Fig. 1). When samples were mixed with standards, dilution curves remained linear indicating that there was no interference from other constituents of the sample (Fig. 1). Sensitivity of the analysis was high; it was possible to measure accurately all the sampled ions in triplicate in only 2 μL of excreta. For convenience of handling, a larger amount (5–10 μL) was normally used for each injection.

IC has been used successfully with a range of plant materials (Masson and Andrieu, 1996), and it offers several advantages over physicochemical methods, particularly for anions (Weiss, 1995). With bulk samples, it is usually necessary to ash the material to remove organic contaminants before IC. This is time-consuming, and it drives off some minerals of interest. With meadow spittlebug excreta, neitherashing nor any other sample preparation was required: The diluted excreta was injected directly onto the IC column. The method is thus rapid and simple. It was also robust: A single pair of IC columns received over 3,000 excreta sample injections with no detectable deterioration in performance. Excreta samples were routinely stored frozen at −20°C for extended periods. Samples stored in this way for over 2 years showed no change in their apparent macronutrient content.

Colorimetric Assay of Ca2+

Where IC is not available or where assay of only a single mineral ion is required, various alternative procedures can be used. An inexpensive colorimetric method was tested here for rapid monitoring of Ca2+ in hundreds of excreta samples. Ca2+ distribution in the xylem is an important factor in many horticultural disorders (Malone and Andrews, 2001). Purification of excreta before the colorimetric assay was not necessary, and Ca2+ concentrations measured in this way correlated well with those obtained by IC (Fig. 2). The meadow spittlebug approach should therefore be accessible to nonspecialist laboratories.

Insect Feeding Patterns

The meadow spittlebug fed throughout the day and night (Fig. 3). Feeding rates were variable, and individuals sometimes showed extended breaks in feeding. However, when plotted against time of day for a population of insects, neither consistent breaks nor any clear diurnal rhythm was apparent (Fig. 3). An increase in feeding activity during the predawn

Figure 1. IC assay performance. A, The chromatogram shows nitrate and phosphate peaks for two sets of dilutions. The peaks have been offset for clarity. One set (thin lines) depicts injections of 1, 2, 3, and 4 mM standard solution. The second set (thick lines) depict injections of the same 1 mM standard, but spiked with 1, 2, 3, and 4 volumes of an excreta sample. B, The graph shows regression lines fitted to peak areas from this chromatogram, and from additional ion peaks in regions of the chromatogram which are not shown. Regression lines for the standard dilutions (white symbols, solid lines) should ideally be straight and pass through the origin. Regression lines for a standard spiked with increasing amounts of sample (black symbols, dashed lines) should similarly be straight and should pass through the points for level 1 of the standard.
period might have been expected, because at this
time, xylem tensions and the energy costs of extrac-
tion are lowest. No such increase was apparent.
The meadow spittlebug is highly polyphagous
(Thompson, 1994), and it fed to some extent on all the
host species that were offered in our experiments.
Besides tomato (Lycopersicon esculentum), pepper
(Capsicum annuum L. cv Bellboy), and broad bean
(Vicia faba), these included sunflower (Helianthus an-
nuus), bean (Phaseolus vulgaris), coleus (Coleus
blumei), wheat, and barley (Hordeum vulgare).

In the greenhouse, feeding rates were about double
on mature pepper plants than on mature tomato
plants. Some insects continued to feed for periods of
8 weeks or more in the greenhouse, but many sur-
vived only for a few weeks. For a cohort of insects
placed simultaneously on mature pepper plants in
the greenhouse, the survival half-time was 9 d.

Evaporation

Evaporation of excreta from the cages was moni-
tored carefully because it could alter the apparent ion
concentration. To reduce evaporation, cages were
wrapped in polythene film (cling film), and the sides
and upper surface of the cages were shaded with
aluminum foil. Evaporation rates were measured by
injecting known amounts of test solutions into blank
cages placed on foliage in the greenhouse, as for the
test cages, and collecting after various times to ascer-
tain how much liquid remained. These tests showed
that the rate of evaporation averaged 1.7 μL h⁻¹
cage⁻¹ (Fig. 4). This was low compared with the rate
of feeding (12–20 μL h⁻¹ cage⁻¹; Fig. 3). When plot-
ted against time of day, evaporation rates showed
some suggestion of an elevation during the after-
noon, but no clear diurnal rhythm (Fig. 4). The influ-
ence of evaporation is reduced by frequent collection
of excreta and by discarding of the smaller samples.
The latter will be more prone to evaporation because
of their greater surface area to volume ratio and
because they come from cages with slow or intermit-
tent feeders. In practice, we collected every 3 to 4 h
during the day and discarded samples under 40 μL.

Ion Content of Meadow Spittlebug

The content of the major mineral cations in
meadow spittlebug tissues was measured after ash-
ing at 550°C and was found to be very low (Table I)
as in many insects (Clark, 1958; Sutcliffe, 1963). Min-
eral cation content of tomato leaf tissue is also shown
in Table I for comparison. The insect’s contents were
much lower than the host shoot except in the case of
Na⁺, which was present at similar levels in both
(Table I).

The daily requirement of these insects for minerals
and water will be extremely small. The adults do
not grow in volume at all. When compared with
the quantities flowing through the insect during
feeding, their bodily requirements will be negligible.
This means that the macronutrient composition of

Figure 2. Colorimetric assay. The Ca²⁺ content of a set of 140
excreta samples was determined using both colorimetric assay and
IC. The line is a fitted regression but forced through the origin.
Without such forcing, the correlation coefficient was 0.9.

Figure 3. Feeding rate with time of day. Accumulation of excreta was
measured periodically for 3 weeks, in six cages on petioles of green-
house peppers. The hourly excretion rate is plotted against time of
day. A different symbol is used for each cage. Each collection interval
spanned several hours, and the point is plotted at the mid-time of the
interval. Two mean accumulation rates are shown (solid lines): The
lower one is the mean for all data. The upper is similar but it excludes
periods of zero feeding.
meadow spittlebug excreta will be practically identical to that of the food source, xylem sap. In the case of K\(^+\), for example, insects feeding on peppers in the greenhouse were found to contain about 5.5 mg g\(^{-1}\) dry weight (Table I). This will overestimate the amount within the insect’s body tissue, because some of this K\(^+\) will be in transit in the gut. Thus, an individual insect of 2.25 mg dry weight will contain up to 12 μg of K\(^+\) in its body tissues. The concentration of K\(^+\) in excreta from these insects is typically about 10 mM (Fig. 5). Thus, when feeding at a normal rate of 20 nL s\(^{-1}\) (Malone et al., 1999), an individual insect will excrete K\(^+\) at about 0.4 ng s\(^{-1}\). Every 30 min, therefore, the insect will void an amount of K\(^+\) equal to that accumulated within its body throughout its entire life (1–4 months). Only a vanishingly small proportion of the K\(^+\) taken in by these insects is retained within the body tissues; virtually all appears in the excreta. Even in the case of Na\(^+\), which is present at lower levels in xylem, an amount equivalent to the insect’s entire body content will be voided every 10 h or so. We can therefore estimate that <1% of the Na in xylem sap is retained by the insect.

Toward the end of the season (October/November), the adult females produce about 20 eggs each (Halkka et al., 1967). The eggs are up to 1 mm long and are produced at a rate of three or four per day in the wild. In the vast majority of cages used here, no eggs appeared, but occasionally two or three were found. Nutrient levels within the eggs were not measured, but unless they contain macronutrients at several orders of magnitude more concentrated than the adults, egg laying will also have negligible impact on levels of ions in excreta.

Mineral anion levels within the insect body were not measured, but the arguments above almost certainly apply to these as well as to the cations. One of the major mineral anions in xylem, nitrate, is not used at all by insects.

The meadow spittlebug will not metabolize any significant proportion of the ammonium in xylem sap, but it could generate a significant amount of ammonium by deamination of dietary amino acids (Andersen et al., 1989). It is difficult to estimate how much amino acid there might be in xylem sap. The arguments of Raven (1983) suggest that, in the extreme case in which all the plant’s organic N is assimilated in the root, the level of amino acid in xylem sap could average some 2.5 mM. If the insect deaminated all of this, 2.5 mM of ammonium could be delivered into the excreta. This would account for most or all of the ammonium observed in excreta.

To test the importance of insect metabolism on the levels of ammonium in excreta in these experiments, xylem sap was extracted from greenhouse peppers by the method of Bollard (1960). This uses mild vacuum to draw fluid from the base of an excised piece of stem, while successively cutting increments from its distal end. The method is entirely destructive but it should produce transpiration-stream fluid with only limited contamination from live cells. There was no difference between the levels of ammonium in this sap and that in excreta from insects feeding on the same plants immediately before excision (data not shown). This indicates that insect metabolism had very little impact on ammonium levels in excreta. Thus, for ammonium as for other minerals, the levels in excreta are very similar to those in xylem sap.

Attempts have been made to analyze more directly the effect of insect metabolism on levels of macronutrient ions in excreta (Ponder et al., 2002). The approaches used by these authors are not without problems, but they support the conclusion that insect metabolism has little or no effect on the level of mineral ions in excreta, even in the case of ammonium.

### Example Data

Figures 5 and 6 illustrate dynamics of macronutrient concentrations in the transpiration stream of in-
tact plants, measured using meadow spittlebug in combination with IC. Results from individual plants are shown, but similar results were obtained simultaneously from neighboring plants. These two data sets come from plants in very different situations: Figure 6 illustrates dynamics of xylem-borne nutrients in a young tomato plant growing in compost in the laboratory, whereas Figure 5 shows the same for a mature pepper plant growing in hydroponic (rockwool) culture in the greenhouse.

Note that samples collected at 8 AM represent material that has accumulated during the night, whereas those collected at 12 PM have accumulated during the morning. The rates of delivery of these various ions to the shoot can be calculated by multiplying the concentration data by the prevailing transpiration rate.

The compost-grown plants (Fig. 6) showed pronounced diurnal rhythms in mineral cations: Xylem K$^+$ was low during the night, but it rose sharply during the morning. Mg$^{2+}$ and Ca$^{2+}$ varied together, also with a pronounced diurnal rhythm, and out of-of-phase with that for K$^+$. Na$^+$ concentration remained low throughout. Among the anions, Cl$^-$ and sulfate varied with a marked diurnal rhythm, which tended to match that for Ca$^{2+}$. Dilution effects arising from the daily rhythm in transpiration cannot account for these patterns. This is because the concentrations of the various ions did not remain constant relative to each other and because there were many intervals during which the concentrations of some ions increased while those of others decreased.

Plants in rockwool were supplied with ample nutrient and had much more K$^+$ and nitrate in their xylem sap (excreta) and much less of other cations and anions (Fig. 5) than did the compost-grown plants. Concentrations of the individual cations (particularly K$^+$, Ca$^{2+}$, and Mg$^{2+}$) tended to vary together in the rockwool plant, and they never moved in opposite directions. The rockwool-grown plants showed no evidence of diurnal rhythms in any of the ions, although there were differences with time. Both sets of plants exhibited a strong diurnal rhythm in transpiration rate (not shown).

The plants compared in Figures 5 and 6 differ in several respects, including species, size, age, and environment. They were chosen to illustrate the versatility of the technique rather than to make compari-

Figure 5. Macronutrient dynamics in a mature pepper growing in rockwool in the greenhouse. The raw data for ion levels in xylem sap (excreta) are shown by white symbols. To highlight trends, a moving average is also plotted for each ion (gray and black symbols). The horizontal markers toward the bottom of each graph indicate periods of night.

Figure 6. Macronutrient dynamics in a young tomato plant growing in compost in the laboratory. Ion levels in xylem sap (excreta) are plotted over a period of several days. The horizontal markers toward the bottom of each graph indicate periods of night.
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MATERIALS AND METHODS

Plant Material

For experiments in the laboratory, seedlings of tomato (Solanum lycopersicum L. Mill cv Ailsa Craig) and broad bean (Vicia faba) were grown to about 25 cm height in 10-cm pots of Levington’s compost. The plants were maintained on a bench by a window, with supplementary lighting of 100 μmol m⁻² s⁻¹ (at plant height) for 14 h d⁻¹ from overhead fluorescent lamps. The midday light level at the leaf surface was typically 200 to 350 μmol m⁻² s⁻¹. Plants were watered every 2nd d but were not given supplementary nutrients.

Figure 7. Diagram of cage assembly. For work in the greenhouse, cages were also covered by cling film and shaded with aluminum foil.

Experiment was also conducted in the greenhouse on mature pepper plants (Capsicum annuum L. cv Bellboy). These were grown in rockwool under semicommercial conditions with a comprehensive nutrient supply (van den Ende et al., 1986) and natural lighting. Their stems were 2 to 3 m in length at the time of these experiments (July–September 1999). For the example data, excreta samples were collected from cages situated midway along the petiole of leaves midway up the plants (about 1.5 m above the root).

Insects

Meadow spittlebug (Philalena spumarius L. [Homoptera: Cercopidae]) adults were collected using a sweep net and aspirator. At our location (52° 12.4’ N 1° 36.1’ W) the nymphs of this insect, conspicuous by their “spittle” masses, appear on a wide variety of host plants from mid-May. The adults appear some 3 weeks later and remain evident until late October. They were locally very common on uncultivated land and, later in the year, on pasture land. Meadow spittlebug adults are about 6 mm long. They exist in several color-pattern morphs and are extremely variable in appearance (Stevens and Lees, 1996). Meadow spittlebug drags the rear pair of legs when walking; this feature is useful in distinguishing it from several generally similar species in the field. Both males and females were collected.

The insects were kept in the laboratory, on 4- to 8-week-old broad bean seedlings, growing in 4 cm of Levington’s compost in a glass aquarium tank of dimensions 30 × 30 × 60 cm. The tank was laid on its side, and the open front of the tank was covered by plastic gauze of mesh size 1 mm. The tank was placed under supplementary lighting as above.

Cage Design

The insects were caged onto petioles at any desired position on the plant. Each cage was made from a 30-mm diameter petri dish (Fig. 7). A pair of diametrically opposed U-shaped notches was cut into the side walls of the petri dish to receive the petiole. A disc of plastic gauze was placed in the bottom of the cage; capillary action along the fibers of this gauze helped to draw all the excreta fluid to a common collection point. The lid of a smaller petri dish (25 mm in diameter) was inverted in the bottom of the cage to hold the gauze flat, and to prevent meadow spittlebug from falling into any accumulated excreta. The cage was secured onto the petiole by a paper clip and elastic band (Fig. 7). When assembled, the single large central hole (approximately 4 mm diameter) in the cage lid was obscured by the elastic band. Smaller holes (approximately 1 mm in diameter) were placed around the periphery of the lid to permit collection of the clear excreta fluid with a Hamilton syringe. Excreta was collected into 0.5-mL Eppendorf tubes, weighed, and stored at −20°C before analysis.

An aspirator was used to add insects through the hole in the cage lid, usually two insects per cage. To reduce evaporation, the entire cage was then enclosed in clear plastic film (cling film), and its top and sides were shaded by aluminum foil.

IC

Excreta samples were thawed, shaken, and centrifuged briefly to pool the tube contents. Ten microliters of excreta (or standard) was typically transferred to a 0.5-mL vial (part no. 038142, Dionex, Camberley, Surrey, UK) and diluted with 300 μL of water or eluent. The vials were then capped, mixed by shaking, and loaded onto an autosampler (AS40, Dionex) attached to a Dionex DX120 IC system. Cation content was determined using an “ionpac” analytical column (CS12A 4 mm) with matching guard column. The eluent for anions was a mixture of 0.4 m Na₂CO₃ and 0.01 m H₂SO₄ made up in ultra pure water (W). The eluent was 20 mH₄SO₄ made up in ultra pure water (>18 MΩ cm⁻¹). Eluent cations were suppressed with a self-regenerating suppressor (CSRS-ULTRA 4 mm), and sample cations were detected by conductivity. Anions were determined in the same way, except using the corresponding anion columns and suppressor. The eluent for anions was a mixture of 0.4 m NaHCO₃ and 5 m Na₂CO₃.

Colorimetric Assay of Ca²⁺

This was conducted in ELISA plates. Three microliters of excreta or standard was added to each well, plus 190 μL of Tris-buffered calcine

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