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# "Afterlife Experiment": Use of MALDI-MS and SIMS Imaging for the Study of the Nitrogen Cycle within Plants

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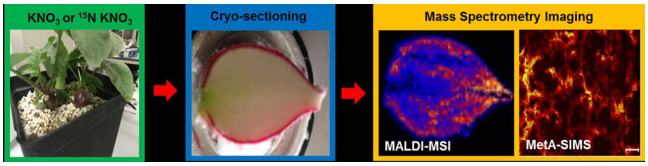
Supporting Information

**ABSTRACT:** As part of a project to demonstrate the science of decay, a series of mass spectrometry imaging experiments were performed. The aim was to demonstrate that decay and decomposition are only part of the story and to show pictorially that atoms and molecules from dead plants and animals are incorporated into new life. Radish plants (*Raphanus sativus*) were grown hydroponically using a nutrient system containing $^{15}N$ $KNO_3$ (98% labeled) as the only source of nitrogen. Plants were cropped and left to ferment in water for 2 weeks to create a radish "tea", which was used as a source of nitrogen for radish grown in a second hydroponics experiment. After 5 weeks of growth, the radish plants were harvested and cryosectioned, and sections were imaged by positive-ion MALDI and SIMS mass spectrometry imaging. The presence of labeled species in the plants grown using $^{15}N$ $KNO_3$ as nutrient and those grown from the radish "tea" was readily discernible. The uptake of $^{15}N$ into a number of identifiable metabolites has been studied by MALDI-MS and SIMS imaging.




In May 2011, the MS Imaging group at Sheffield Hallam University was approached by the BBC to take part in a project to demonstrate the science of decay.[1] The project was set up in Edinburgh Zoo in the summer of 2011; a fully equipped kitchen and garden together with all the detritus from a family barbeque was sealed in a glass box. The program was a joint venture between the BBC and the Discovery Channel and closely followed the events as maggots, molds, bacteria, flies, and mushrooms transformed the contents beyond all recognition. As part of the experiment, the program makers wished to demonstrate that decay and decomposition was only part of the story and that atoms and molecules from dead plants and animals can be incorporated into new life.

The element nitrogen is a ubiquitous component of all living systems and is located, primarily, in amino acids, proteins, nucleic acids, and in the chlorophyll of plants. Nitrogen is one of the principal nutritional requirements of all plants which acquire it through their root systems, usually in the form of nitrates. In the plant, the nitrate is quickly converted into nitrites and then ammonium ions, which can be assimilated into amino acids.[2] The nitrogen cycle has been extensively documented. Research into the nitrogen cycle first started in the 19th century, with scientists such as Boussingault shaping modern agrochemistry.[3]

There have been many studies of the uptake and metabolization of nitrogen by plants, and many of these utilize the isotope $^{15}N$; this is a rare but stable isotope, with a natural abundance of 0.366% (0.00366 mole fractions).[4,5] Plant studies utilizing $^{15}N$-enriched $KNO_3$ material as a tracer in isotopic analysis are well-documented, with techniques such as gas chromatography-mass spectrometry (GC-MS),[6] inductively coupled plasma-optical emission spectroscopy (ICP-OES),[7] microwave induced plasma-optical emission spectroscopy (MIP-OES),[8] nuclear magnetic resonance (NMR),[9] liquid chromatography-tandem mass spectrometry (LC-MS/MS),[6] multi-isotope imaging mass spectrometry (MIMS),[10] and secondary ion mass spectrometry (SIMS)[11] all being employed. Furthermore, the tracer been used in agricultural research to trace mineral nitrogen compounds (particularly fertilizers) in the environment and is also very important for describing the fate of nitrogenous organic pollutants.[12,13]

Matrix-assisted laser/desorption ionization-mass spectrometry imaging (MALDI-MSI) is an emerging technique within plant biology.[14] In the past, MALDI-MSI has been used to illustrate the distribution of various plant metabolites,[15−17] including carbohydrates,[18] oligosaccharides,[19] proteins,[20,21] lipids,[22,23] peptides,[24] pesticides,[25] and various agrochemicals[26] within plant tissue. Until now, the use of this technique to study nutrient cycles within plants has not been described. The aim of the work reported here is to demonstrate that mass spectrometry imaging is a valuable tool in the tracking of the distribution of $^{15}N$-labeled compounds and, specifically for the purpose of the BBC project, that it can be used to show the uptake of species from dead organisms by living ones (i.e., to study nutrient cycles).

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## EXPERIMENTAL SECTION

**Materials.** Alpha-cyano-4-hydroxycinnamic acid (CHCA), choline chloride, 99%, 4-aminobutyric acid, 99+%, methanol, acetonitrile, and trifluoroacetic acid (TFA) (AR grade) were purchased from Fisher Scientific (Loughborough, U.K.). Calcium chloride, calcium sulfate, magnesium sulfate, potassium phosphate, and a trace element mix were all purchased from Hortifeeds (Lincoln, U.K.). 2,5-Dihydroxybenzoic acid (DHB), potassium nitrate-$^{15}$N 98 atom % $^{15}$N ($^{15}$N-$KNO_3$), and potassium nitrate *ReagentPlus* ≥99.0% were purchased from Sigma-Aldrich (Gillingham, U.K.).

**Cultivation (Hydroponic Experiment).** Two groups of radish seeds (*Raphanus sativus*) were sown in a mix of perlite and vermiculite (4:3) in an artificially controlled environment. The control group utilized a nutrient system containing standard $KNO_3$ and the first generation $^{15}$N group replaced all of the $KNO_3$ with $^{15}$N $KNO_3$ (98% labeled) as the only source of nitrogen. Plants were cropped, homogenized, and left to ferment in ultrapure water (18.2 MΩcm) obtained from a Purelab ultra water purification system (Elga, Ransbach-Baumbach, Germany) for 2 weeks to create a "tea". The "tea" was used as the only source of nitrogen for a second hydroponics experiment (second generation $^{15}$N group) where radishes were grown in an artificially controlled environment. After 5 weeks of growth, the radish plants were harvested. Samples of leaves, bulbs, and stems from each group (control, first generation $^{15}$N and second generation $^{15}$N) were cryosectioned, and sections were imaged by positive-ion MALDI and SIMS imaging (Figure 1).

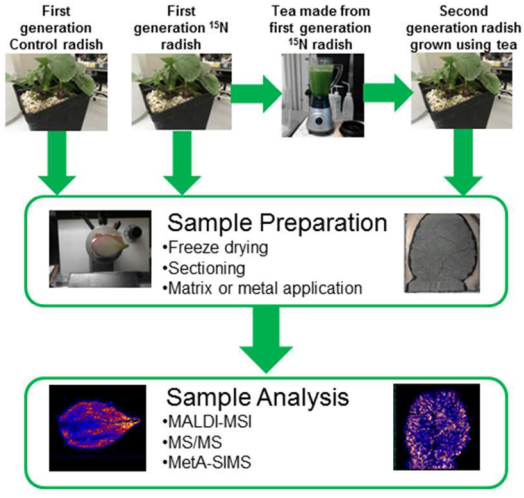


**Figure 1.** Experimental workflow used in this study.

**Sample Preparation.** The radishes were harvested, and the bulbs were separated from the roots and leaves and snap frozen in liquid nitrogen. The frozen radish bulbs were sectioned at a temperature of −20 °C using a Leica Cryostat (Leica, Wetzlar, Germany) to obtain 12 μm sections, which were thaw mounted onto glass slides and indium tin oxide (ITO) coated glass slides (4−8 Ω resistance, Delta Technologies, Stillwater, MN, U.S.A.). Leaves were prepared by placing fresh samples between tissue paper and pressing between two glass slides. The slides were taped together and placed into a freeze drier at −36 °C until they reached a constant mass, approximately 10 days. The dried leaves were mounted onto either aluminum target plates or glass slides using double sided carbon tape to adherer the sample.

**MALDI Matrix Deposition.** Samples for MALDI were coated with a 5 mg/mL solution of CHCA in 70:30 methanol/water with 0.2% TFA using the Suncollect automated pneumatic sprayer (Sunchrom GmbH, Friedrichsdorf, Germany) in a series of 10 layers. The initial seeding layer was performed at 2 μL/min and subsequent layers were performed at 3 μL/min. Some samples were coated with a 20 mg/mL solution of DHB in 50:50 acetonitrile/water with 0.2% TFA using the Suncollect automated pneumatic sprayer in a series of 30 layers. The initial seeding layer was performed at 10 μL/min then stepped up to 20 μL/min, and subsequent layers were performed at 30 μL/min.

**SIMS Sample Gold Coating.** Prior to analysis, the radish bulb sections were gold-coated using a Quorum Technologies SC7640 sputter coater (New Haven, U.S.A.) equipped with a FT7607 quartz crystal microbalance stage and FT690 film thickness monitor to deposit a 1 nm thick gold layer.

**Instrumentation.** *Applied Biosystems Q-Star Pulsar I.* Initial mass spectrometric analyses were performed using a Applied Biosystems API Q-Star Pulsar i hybrid quadrupole time-of-flight (QTOF) instrument (Concord, Ontario, Canada) fitted with an orthogonal MALDI ion source and a 1 kHz Nd:YAG solid-state laser. Images were acquired at a spatial resolution of 150 μm × 150 μm in "raster image" mode,[27] using "oMALDI Server 5.1" software supplied by MDS Sciex (Concord, Ontario, Canada) and generated using the freely available Biomap 3.7.5.5 software (Novartis, Basel, Switzerland). Mass spectra were processed either in Analyst MDS Sciex (Concord,Ontario,Canada) or using the open source multifunctional mass spectrometry software mMass.[28]

MALDI-MS spectra were obtained in positive-ion mode in the mass range between $m/z$ 50 and 1000. Declustering potential 2 was set at 15 arbitrary units and the focus potential at 10 arbitrary units, with an accumulation time of 0.999 s. Average spectra were acquired over a 0.5 $cm^2$ region on the leaves. The MALDI-MS/MS spectrum of the unknown precursor ions at $m/z$ 104.1 and 105.1 was obtained using argon as the collision gas; the declustering potential 2 was set at 15 and the focusing potential at 20, and the collision energy and the collision gas pressure were set at 20 and 5 arbitrary units, respectively.

*Waters Synapt G2 HDMS.* Further MALDI data were acquired using a Waters MALDI SYNAPT G2 HDMS mass spectrometer (Waters Corporation, Manchester, U.K.) to acquire mass spectra and images. Prior to MALDI-MSI analysis, the samples were optically scanned using a flatbed scanner to produce a digital image for future reference, this image was then imported into the MALDI imaging pattern creator software (Waters Corporation) to define the region to be imaged. The instrument was calibrated prior to analysis using a standard mixture of polyethylene glycol. The instrument was operated in V-mode and positive-ion mode, and all data were acquired in the mass range of $m/z$ 100 to 500. The data were then converted using MALDI imaging converter software (Waters Corporation) and visualized using the BioMap 3.7.5.5 software (Novartis, Basel, Switzerland).

*MetA-SIMS Imaging.* SIMS data were acquired using a Physical Electronics TRIFT II TOF-SIMS (Physcial Electronics, U.S.A.) equipped with an Au liquid metal gun tuned for 22 keV Au+ primary ions. Images were acquired in mosaic mode using 64 tiles that each measures 125 μm square and


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contains 256 × 256 pixels. The total area scanned was 1 × 1 mm area with 60 s/tile resulting in a total of 4 194 304 spectra.

*Data Processing.* Images from the Q-Star instrument were processed using Biomap 3.7.5.5 software. All images were normalized against the total ion count (TIC). For presentation purposes, the mass spectra from the Analyst QS 1.1 software were exported in the form of a text file and then imported into the mMass software for processing.

MALDI-MSI data from the Waters Synapt instrument were converted into Analyze 7.5 format using MALDI imaging converter software (Waters Corporation) and visualized using the BioMap 3.7.5.5 software (Novartis, Basel, Switzerland). SIMS data were analyzed and visualized using WinCadence software version 4.4.0.17 (Physical Electronics).

## RESULTS AND DISCUSSION

Previous work has demonstrated that plants quickly metabolize nitrogen into amino acids after assimilation via the roots.[2] Initially, glutamine and glutamate were investigated, as these are the primary amino acids formed from nitrogen uptake. Other amino acids were also investigated with particular attention to arginine ($m/z$ 175), as previous work using MALDI-MSI had demonstrated its distribution within plants.[16] The focus on these particular amino acids was intended to demonstrate the incorporation of nitrogen from the now dead radish plants into protein synthesis for the new living plants; however unambiguous identification of these amino acids proved to be difficult because of the complex overlapping isotope peaks present in this region of the positive-ion mass spectrum.

However, the B-complex vitamin choline, ($C_5H_{14}NO$), $m/z$ 104.1, was found to have incorporated the labeled $^{15}N$, producing an ion at $m/z$ 105.1. These data are presented as Figures 2 and 3. Further identification of these peaks as arising from choline is supported by the MALDI-MS/MS data presented in Figure S1. Figure S1 confirms the identification of the $m/z$ 104 and $m/z$ 105 ions as choline and $^{15}N$-labeled choline, respectively, clearly showing the appropriate product ions. The spectrum produced matched data generated by the Scripps Centre for Metabolomics. Also, the product ions that contain nitrogen from the $^{15}N$ radish bulb and leaves have all increased in mass-to-charge by one, Figure S1b, which further confirms the identification.

The main distribution of the choline within the bulbs was found to be near to the skin, but not actually within the skin. It was also widely distributed through the bulb (Figure 2). This is supported by the distribution of pelargonidin ($m/z$ 271). This is a natural food coloring found in radish skin, and when overlaid with the choline, it can be clearly seen that these species exist in two distinct and separate areas (Figure 4).

The MALDI-MS images in Figure 2 show the full extent of the distribution of the labeled and the unlabeled choline. The MALDI-MS spectra of the three leaves in Figure 3 also reveal the difference in the intensity of the labeled ($m/z$ 105) and unlabeled ($m/z$ 104) choline. Figure 3a shows a higher intensity of the unlabeled choline ($m/z$ 104), whereas 3b shows a higher intensity of labeled choline ($m/z$ 105), confirming that the $^{15}N$ was metabolized by the plant and incorporated into its structure. Figure 3c demonstrates that the $^{15}N$ was then passed from the first generation to the second generation radish that was grown with plant material that had been feed exclusively $^{15}N$ nitrogen. The even intensity and distribution of labeled and unlabeled choline can be clearly seen in Figures 2 and 3c.

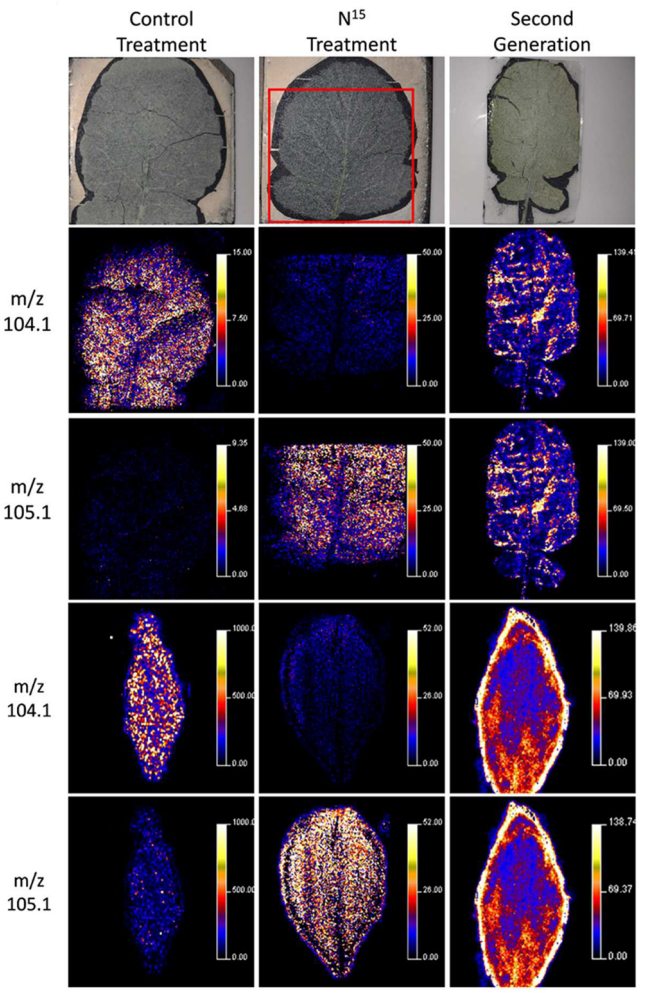


**Figure 2.** MALDI-MS images showing the distribution of choline at $m/z$ 104 and 105 within the leaf and the bulb of the radish (normalized against the TIC).

The MALDI-MS images displayed in Figure 4 show the distribution of labeled and unlabeled species in the second generation radish bulb section, following normalization against the total ion current (TIC). The species at $m/z$ 271 (Figure 4a) appears to be exclusively localized within the skin of the radish bulb section. This has been tentatively identified as the anthocyanidin pelargonidin, which is the natural food color in the radish skin.[29] In order to confirm this assignment, MS/MS was performed, and the resulting spectrum Figure S2 matched that of the reference MS/MS data independently generated by the Scripps Center for Metabolomics.

The distributions of the unlabeled and labeled choline at $m/z$ 104 and 105 (Figure 4b,c, respectively) are predominantly in the center of the radish bulb. This is also observed with unlabeled and labeled phosphocholine at $m/z$ 184 and 185 (Figure 4d,e, respectively). The distributions of labeled choline and phosphocholine at $m/z$ 105 and 185 were overlaid with the distribution of pelargonidin at $m/z$ 271, which is shown in Figure 4f,g. These images demonstrate that there is a clear distinction between the two regions and that the labeled species are only present in the center of the radish.

The MALDI-MS spectrum (Figure 3C) shows that approximately 50% of the material from the first generation radishes has successfully been transferred and assimilated into


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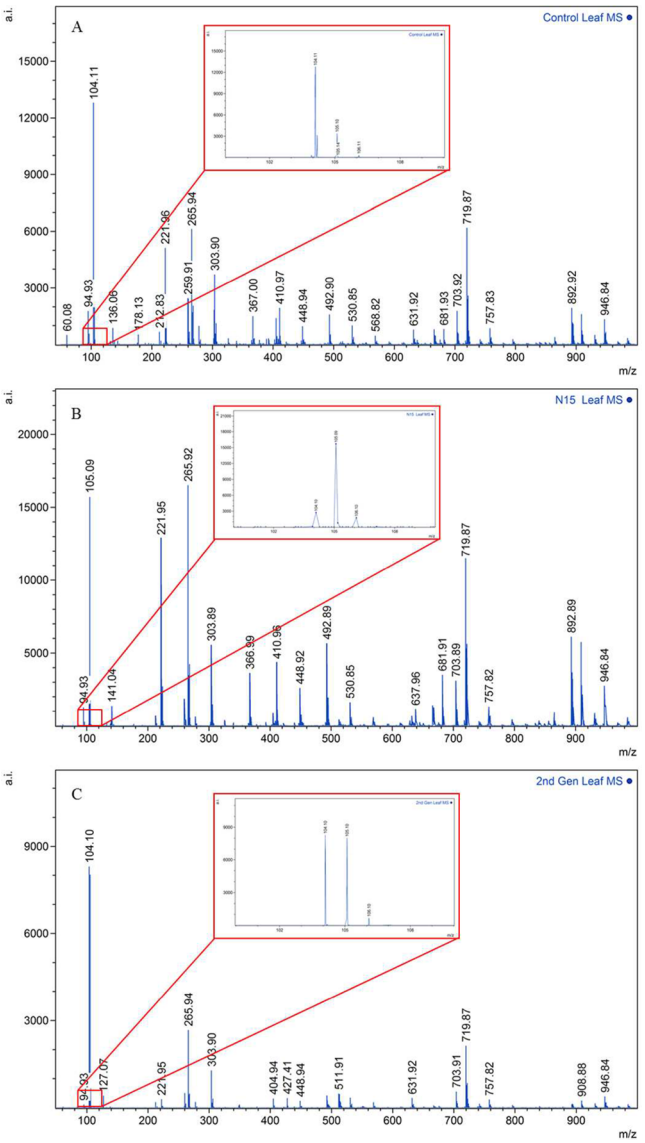


**Figure 3.** MALDI-MS spectra obtained from the leaves of the (a) control, (b) $^{15}$N radish, and (c) second generation $^{15}$N plant.

the formation of the second generation radish. This is also visually reflected in the MALDI-MS images shown in Figures 2 and 4 which shows the same distribution with equal intensity for the labeled and unlabeled choline and phosphocholine.

The metal assisted-secondary ion mass spectrometry (MetA-SIMS) images of the control radish bulb section are displayed in Figure 5. The optical image (Figure 5a) shows a magnified view of the center portion of the radish, which looks like that of the first generation radish. The TIC image is shown in Figure 5b. The MetA-SIMS images of the control radish show the distribution of unlabeled choline at $m/z$ 104 (Figure 5c) and the isotope of this peak at $m/z$ 105 (Figure 5d). The mass spectrum (Figure 5e) shows a strong peak at $m/z$ 104 which has been tentatively assigned to the unlabeled choline. This is expected to be the abundant form because this radish was grown exclusively with an unlabeled compound. The isotopic ratio ($m/z$ 104/ $m/z$ 105) for the control radish is 2.5322.

The MetA-SIMS images displayed in Figure 6 shows the distribution of labeled and unlabeled choline in the first generation radish bulb section. The optical image (Figure 6a) shows a magnified view of the center portion of the radish, and the TIC image is shown in Figure 6b. The MetA-SIMS images of unlabeled choline at $m/z$ 104 and $^{15}$N labeled choline at $m/z$ 105 (Figure 6c,d, respectively) show both species are widely distributed in the string like network observed in the optical image. The related spectrum (Figure 6e) shows a strong peak at $m/z$ 105 which could be $^{15}$N-labeled choline, which is expected as this radish was grown exclusively with a $^{15}$N-labeled compound. The isotopic ratio for the first generation radish was calculated from the SIMS data to be 0.1963.

The MetA-SIMS images of the second generation radish bulb section displayed in Figure 7 shows the distribution of labeled and unlabeled choline. The optical image (Figure 7a) shows a magnified view of the center portion of the radish. The TIC image is shown in Figure 7b. The MetA-SIMS images of unlabeled choline at $m/z$ 104 (Figure 7d) and $^{15}$N-labeled choline at $m/z$ 105 (Figures 7c) show both species are evenly distributed through the imaged area. The related spectrum (Figure 7e) again shows a peak at $m/z$ 105 which could be $^{15}$N-labeled choline; however, neither the images nor the spectrum reflect the same pattern previously observed in the MALDI-MS data of the second generation radish. The isotopic ratio calculated for the second generation radish is 0.4689. This variation is probably due to the MetA-SIMS data only representing a very small portion of a large sample, and hence, it shows the local uptake of the $^{15}$N label and its incorporation into choline. This is in contrast to the MALDI-MS image (Figure 4), which shows the global distribution of labeled and unlabeled choline throughout the whole radish bulb section.

Previous studies using isotopes such as $^{14}$C, $^{15}$N, and $^{31}$P have demonstrated the cycle of these key nutrients for the plant.[5−8,12,13] Techniques such as inductively coupled plasma-mass spectrometry (ICP-MS),[10,30] GC-MS[6], ICP-OES[7], MIP-OES[8], NMR[9], and LC-MS/MS[6] have been employed in the study of nutrient cycles and have all been used to demonstrate the nitrogen cycle. However, employment of these techniques only gave a general distribution of the nitrogen within the tissue, and in addition, each of these methods require the tissue to be destroyed either by digestion or extraction methods in order to be analyzed. Imaging techniques have been used to study plants extensively including laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS),[15,31−33] X-ray absorption spectroscopy (XAS),[31,33] X-ray fluorescence (XRF),[31,33] SIMS,[10,15,31,33] and MALDI-MS,[14−17,20,22,23] but the data presented here is the first use of mass spectrometry to demonstrate unambiguously that atoms and molecules move from dead plant material into new living material.

This is also the first demonstration of the use of MALDI-MS and SIMS imaging to show the distribution, translocation, and metabolism of nitrogen in plants. MALDI-MS imaging of plant material is a relatively new technique, with only a small number of researchers performing it. Compounds such as nicosulfuron[25] and other agrochemicals compounds[26] have previously been monitored using this technique, but no demonstration of the cycle of this compound was performed. Other naturally occurring compounds in plants to be monitored and mapped using MALDI-MS imaging include amino acids,[14,16,34,35] oligosaccharides,[19] carbonhydrate,[18] proteins,[20] peptides,[24] lipids,[22,23] and other plant metabolites.[15−17] The technique has also been proven to be useful in the monitoring of metabolites of nitrogen fixing bacteria known as rhizobia.[36] MS imaging of stable-isotope-labeled compounds will therefore


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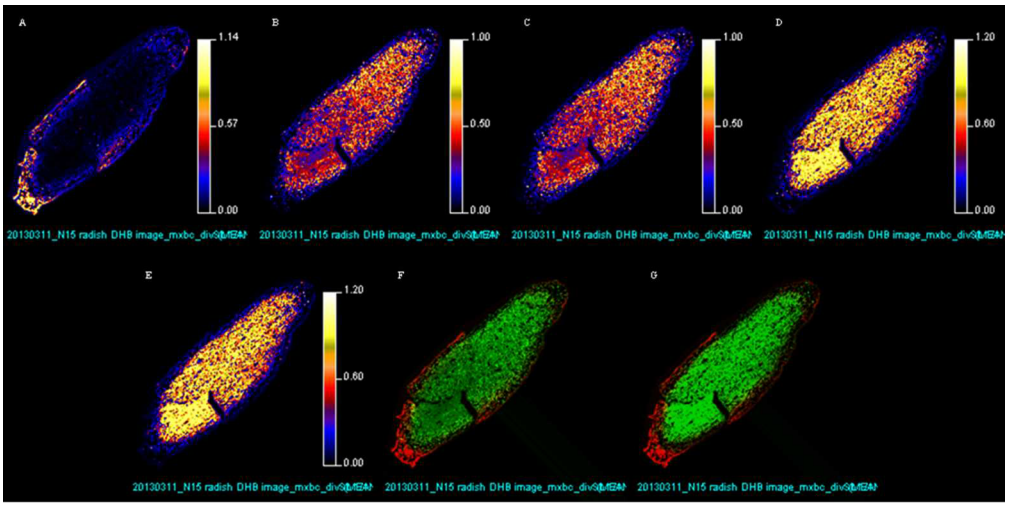


**Figure 4.** MALDI-MSI images showing the distribution of (A) a species identified as the anthocyanidin pelargonadin at $m/z$ 271, (B) choline at $m/z$ 104, (C) $^{15}$N-labeled choline at $m/z$ 105, (D) phosphocholine at $m/z$ 184, (E) $^{15}$N-labeled phosphocholine at $m/z$ 185, (F) $^{15}$N-labeled choline at $m/z$ 105 overlaid onto the unknown species at $m/z$ 271, and (G) $^{15}$N-labeled phosphocholine at $m/z$ 185 overlaid onto pelargonadin species at $m/z$ 271.

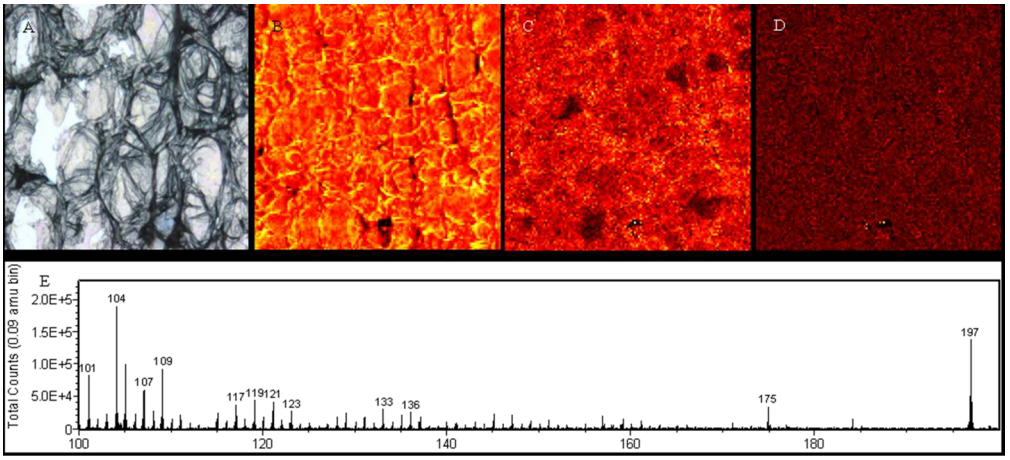


**Figure 5.** (A) Optical image of the control radish bulb section and MetA-SIMS images showing (B) the total ion current, the distribution of (C) choline at $m/z$ 104 and (D) C13 isotope of choline at $m/z$ 105, and (E) MetA-SIMS spectrum obtained from the radish bulb section.

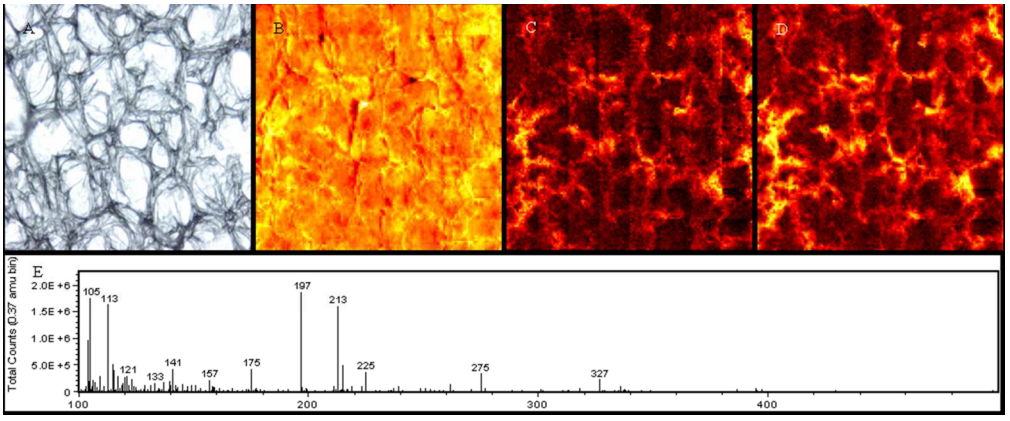


**Figure 6.** (A) Optical image of the first generation radish bulb section and MetA-SIMS images showing (B) the total ion current, the distribution of (C) choline at $m/z$ 104 and (D) $^{15}$N-labeled choline at $m/z$ 105, and (E) MetA-SIMS spectrum obtained from the radish bulb section.

clearly be a useful tool in the future for monitoring the distribution of other species within plants, because this technique could be used to monitor the uptake and distribution of herbicides, pesticides, and plant growth regulator (PGR) not only within the primary plant they were applied but also in the second generation plant. It could also be used to monitor bioremediation and show if plants have been grown on contaminated land. Furthermore, it has the potential to help


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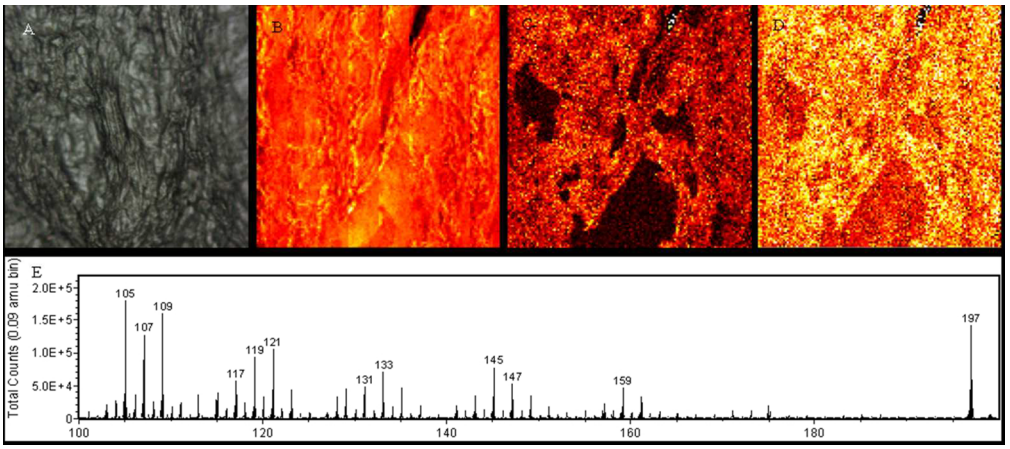


**Figure 7.** (A) Optical image of the second generation radish bulb section and MetA-SIMS images showing (B) the total ion current, the distribution of (C) $^{15}$N-labeled choline at $m/z$ 105 and (D) choline at $m/z$ 104, and (E) MetA-SIMS spectrum obtained from the radish bulb section.

optimize plant nutrition, nutrient delivery, and schedules. Previous work performed by Bateman and Kelly,[37,38] which employed a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20−20 isotope ratio mass spectrometer for the analysis of fertilizers, could be extended to confirm that the nitrates taken up by the second generation plant were either from inorganic nitrates released from the first generation plants or organic nitrogen realized into the tea.

## CONCLUSION

The data presented here have demonstrated that atoms and molecules move from dead plant material into new living material and that MALDI-MS and TOF-SIMS imaging are useful tools for monitoring the distribution of compounds within plants and that the use of stable isotope labeled compounds in MS imaging experiments can yield useful information about uptake and fate in metabolomics-based experiments.

Mass spectrometry imaging has been used to demonstrate the uptake of labeled nitrogen species from composted radish leaves into growing radish plants. The "cycle" of life has been successfully demonstrated.

## ASSOCIATED CONTENT

### Supporting Information

Figures S1 and S2 are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org/.

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

(1) BBC Four, AfterLife: The Strange Science of decay. http://www.bbc.co.uk/programmes/b012w66t (Accessed November 2013).
(2) Scott, P. *Physiology and Behaviour of Plants*; John Wiley & Sons Ltd: Chichester, U.K., 2008.
(3) Keeney, D. R.; Hatfield, J. L.; Follett, R. F., Hatfield, J. L., Eds.; In *Nitrogen in the Environment: Sources, Problems and Management*; Elsevier Science: Amsterdam, 2001; pp 3−16.
(4) Berglund, M.; Wieser, M. E. *Pure Appl. Chem.* **2011**, *83*, 397−410.
(5) Dawson, T. E.; Mambelli, S.; Plamboeck, A. H.; Templer, P. H.; Tu, K. P. *Annu. Rev. Ecol. Syst.* **2002**, *33*, 507−559.
(6) Engelsberger, W. R.; Erban, A.; Kopka, J.; Schulze, W. X. *Plant Methods* **2006**, *2*, 14.
(7) Fiedler, R.; Proksch, G. *Plant Soil* **1972**, *36*, 371−378.
(8) Heltai, G.; Jozsa, T. *Microchem. J.* **1995**, *51*, 245−255.
(9) Knicker, H.; Lüdemann, H. *Org. Geochem.* **1995**, *23*, 329−341.
(10) Lechene, C. P.; Luyten, Y.; McMahon, G.; Distel, D. L. *Science* **2007**, *317*, 1563−1566.
(11) Grignon, N.; Halpern, S.; Jeusset, J.; Briancon, C.; Fragu, P. *J. Microsc.* **1997**, *186*, 51−66.
(12) Marsh, K. L.; Sims, G. K.; Mulvaney, R. L. *Biol. Fertility Soils* **2005**, *42*, 137−145.
(13) Bichat, F.; Sims, G. K.; Mulvaney, R. L. *Soil Sci. Soc. Am. J.* **1999**, *63*, 100−110.
(14) Kaspar, S.; Peukert, M.; Svatos, A.; Matros, A.; Mock, H. *Proteomics* **2011**, *11*, 1840−1850.
(15) Lee, Y. J.; Perdian, D. C.; Song, Z.; Yeung, E. S.; Nikolau, B. J. *Plant J.* **2012**, *70*, 81−95.
(16) Burrell, M. M.; Earnshaw, C. J.; Clench, M. R. *J. Exp. Bot.* **2007**, *58*, 757−763.
(17) Li, Y.; Shrestha, B.; Vertes, A. *Anal. Chem.* **2008**, *80*, 407−420.
(18) Robinson, S.; Warburton, K.; Seymour, M.; Clench, M.; Thomas-Oates, J. *New Phytol.* **2007**, *173*, 438−444.
(19) Wang, J.; Sporns, P.; Low, N. H. *J. Agric. Food Chem.* **1999**, *47*, 1549−1557.
(20) Grassl, J.; Taylor, N. L.; Millar, A. H. *Plant Methods* **2011**, *7*, 21.
(21) Schwartz, S. A.; Reyzer, M. L.; Caprioli, R. M. *J. Mass Spectrom.* **2003**, *38*, 699−708.
(22) Vrkoslav, V.; Muck, A.; Cvacka, J.; Svatos. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 220−231.
(23) Horn, P. J.; Korte, A. R.; Neogi, P. B.; Love, E.; Fuchs, J.; Strupat, K.; Borisjuk, L.; Shulaev, V.; Lee, Y.; Chapman, K. D. *Plant Cell* **2012**, *24*, 622−636.
(24) Kondo, T.; Sawa, S.; Kinoshita, A.; Mizuno, S.; Kakimoto, T.; Fukuda, H.; Sakagami, Y. *Science* **2006**, *313*, 845−848.
(25) Anderson, D. M. G.; Carolan, V. A.; Crosland, S.; Sharples, K. R.; Clench, M. R. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1321−1327.
(26) Mullen, A. K.; Clench, M. R.; Crosland, S.; Sharples, K. R. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2507−2516.

(27) Trim, P. J.; Djidja, M.; Atkinson, S. J.; Oakes, K.; Cole, L. M.; Anderson, D. M. G.; Hart, P. J.; Francese, S.; Clench, M. R. *Anal. Bioanal. Chem.* **2010**, *397*, 3409−3419.
(28) Strohalm, M.; Kavan, D.; Novak, P.; Volny, M.; Havlicek, V. *Anal. Chem.* **2010**, *82*, 4648−4651.
(29) Wu, X. L.; Prior, R. L. *J. Agric. Food Chem.* **2005**, *53*, 3101−3113.
(30) Mihaylova, V.; Lyubomirova, V.; Djingova, R. *Int. J. Environ. Anal. Chem.* **2013**, *93*, 1441−1456.
(31) Lombi, E.; Scheckel, K. G.; Kempson, I. M. *Environ. Exp. Bot.* **2011**, *72*, 3−17.
(32) Wu, B.; Zoriy, M.; Chen, Y.; Becker, J. S. *Talanta* **2009**, *78*, 132−137.
(33) Wu, B.; Becker, J. S. *Metallomics* **2012**, *4*, 403−416.
(34) Gogichaeva, N. V.; Alterman, M. A. *Methods Mol. Biol.* **2012**, *828*, 121−35.
(35) Gogichaeva, N. V.; Williams, T.; Alterman, M. A. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 279−284.
(36) Ye, H.; Gemperline, E.; Venkateshwaran, M.; Chen, R.; Delaux, P.; Howes-Podoll, M.; Ane, J.; Li, L. *Plant J.* **2013**, *75*, 130−145.
(37) Bateman, A. S.; Kelly, S. D.; Jickells, T. D. *J. Agric. Food Chem.* **2005**, *53*, 5760−5765.
(38) Bateman, A. S.; Kelly, S. D. *Isot. Environ. Health Stud.* **2007**, *43*, 237−247.


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