315 Title: Methods for measuring nutrient uptake in maize using nitrogen stable isotopes.

316 Findimila Dio Ishaya[1], Amanda Rasmussen[1*]

317

318 [1] School of Biosciences, Division of Agriculture and Environmental Sciences, University of

319 Nottingham, Sutton Bonington, UK LE12 5RD

320

321 [*]Corresponding author: Amanda.rasmussen@nottingham.ac.uk

322

323

324

325

326

327

328

329

330

331

332

333

334

ABSTRACT

Understanding nutrient uptake is central to improving nitrogen use efficiency in crops, including maize. Reducing the need for fertiliser without reducing yield is extremely important, as nitrogen fertilisers come with a high environmental cost, both in terms of emissions from manufacturing and in losses to waterways or volatilisation off fields. Maize develops multiple different root types including primary, seminal, crown and brace roots. Part of improving efficiency in maize is understanding the differences in nutrient uptake via each distinct root type, but these differences have been largely ignored to date. Here, we describe a protocol that uses stable isotopes for determining nitrogen uptake rates by maize root types. We describe the steps both for intact roots, for which we use rhizoboxes with openable front windows that allow access to the roots without disturbing the rest of the plant, and for field-grown plants, for which intact analysis is not feasible and requires excising the roots. The methods described here can also be modified to measure uptake kinetics and for monitoring nutrient translocation between roots and shoots. Improving our understanding of root physiology and nutrient dynamics via these methods will improve breeding opportunities for efficient nutrient uptake varieties, reducing the need for fertiliser additions.

Keywords: nitrogen; fertiliser; stable isotopes; nutrient

INTRODUCTION

Before we discuss nitrogen uptake by the roots, we first need to describe the maize root system, which contains multiple distinct root types that are subjected to different developmental controls. The maize root system comprises embryonic (primary and seminal) and postembryonic (brace, crown, and lateral) roots (Hochholdinger et al. 2004; Hochholdinger et al. 2018a; Hostetler et al. 2021). The embryonic root system consists of a single primary root, and a variable number of seminal roots formed at the scutella node that are important for seedling vigour during early development. The postembryonic root system consists of roots formed from consecutive shoot nodes (nodal roots), and include crown roots from below ground nodes and brace roots from above ground nodes. Finally, postembryonic lateral roots are initiated in the pericycle of all root types (Hochholdinger et al. 2004; Saengwilai et al. 2014; Yu et al. 2015; Hochholdinger et al. 2018b; Yu et al. 2018).

Since plants are continuously taking up nutrients, studying nutrient uptake requires spiking the roots with a trackable molecule, one that is distinct from the one of interest but similar enough to replicate the process. The best way to do this is by using stable isotopes that are naturally present at very low levels.

Here, we describe a protocol for ^{15}N tracking on both intact plants and on excised roots of different types (i.e. crown, seminal, and brace) from field-grown plants. Briefly, the workflow involves selecting plants, preparing stable isotope solutions (usually the day before the treatments, one per nitrogen source to test), applying ^{15}N to the roots, rinsing, imaging and drying, grinding, weighing into tin capsules, sending to a laboratory service? for quantification of ^{15}N, and data analysis. Where possible, we describe ways to optimise the workflow during waiting steps or when additional staff are available.

There are subtle differences between the protocols for intact or excised roots. In the approach described here, the main differences relate to sourcing roots and handling the roots as they go into treatments (Steps 15-17 for intact and Steps 18-21 for field-grown excised), which is why there are

separate subheadings. Intact systems for example using rhizoboxes, allow access to roots of known age and depth in the soil. In contrast, it is difficult to know where or how large each root type is on field grown plants. Good practice would involve an initial test by growing plants in a field and digging a few up every few days to get an indication of how fast each root type grows. Brace roots, however, are much easier in this context, as they start above ground. Field-grown plants are also very difficult to keep undamaged while treating the roots and, so, excavation followed by root excision is usually the only practical option. The advantage of field grown plants is that they are closer to normal field cropping systems. It needs to be remembered that uptake measurements on excised roots underestimates the uptake of an intact root system (Brackin et al. 2015). For both intact and field-grown systems, the brace roots emerge much later than seminal or crown roots. This means that comparisons will either use plants of one developmental stage but roots of a different age (and probably different depths in the soil) or will compare root types of the same age (and depth) but the plants will be at different developmental stages.

We describe here how to test nitrogen uptake capacity and nitrogen source preference. This involves feeding each root a mixture of nitrogen sources, with one of those sources labelled with ^{15}N in each. If one is interested in assessing only a single nitrogen source, then the protocol below can be adapted such that only a single nitrogen mixture containing the labelled ^{15}N-type of interest is used. Another adaptation is to study uptake kinetics, which requires a single label at a range of concentrations, each concentration fed to each root (for example Brackin et al. 2015). Again, the protocol described here can be changed to a single nitrogen source but multiple concentrations of the labelled source.

The protocol assumes users have considered their research question and already have growing plants either in a greenhouse or field setting and are ready to perform to nutrient uptake assays.

MATERIALS

It is essential to check the general Material Data Sheet and your institutions health and safety procedures for proper handling and use of equipment used in this protocol.

Reagents

Plants from which roots will be sampled

^{15}N enriched Sources

While any level of ^{15}N enrichment (percentage of ^{15}N in the source) can be used, it is important to know the level in your source. The information on enrichment is available from the suppliers and will usually be written on the bottle. It is important to ensure that all treatments have the same level of enrichment. You will see in the source list below that our glycine has a higher enrichment, so we dilute it with unlabelled glycine (see Step 2).

^{15}Glycine (e.g. Sigma Aldrich, 299294 ; *98% enrichment*)

K^{15}-labeled potassium nitrate (K$^{15}NO_3$) (e.g. Sigma Aldrich, 348481 ; *10% enrichment)*

^{15}N-labeled ammonium sulfate ($(^{15}NH_4)_2SO_4$) (e.g. Sigma Aldrich, 348473; *10% enrichment)*

Non-^{15}N enriched Sources

Ammonium sulfate ($(NH_4)_2SO_4$)

Glycine

Potassium nitrate (KNO_3)

Potassium chloride (KCl)

Water (MiliQ)

Equipment

Borosilicate glass laboratory reagent bottles with lid, for mixing solutions

Ideally have three, one for each mixture containing each ^{15}N label, to avoid any risk of cross contamination of ^{15}N labels. The sizes of the bottles depends on the number of samples.

Box (large cardboard box in which to put the sample bags)

Buckets and hose for root washing field-grown plants after excavation

Bucket with rubbish bag to put finished Falcon tubes

Camera

Blue background with ruler

A blue plastic folder or clipboard works well as it can be wiped dry between photos.

Ceramic mortars (60-100 mm in diameter) and pestles

Having as *many as possible available helps with the speed of grinding before they all need thorough cleaning*

Eppendorf tubes (1.5-mL)

Ground samples are transferred to the Eppendorf tubes for storage.

Falcon tubes (15-mL) and racks to support and store the tubes. *Prepare twice as many tubes as roots are being sampled – one for labelled mixture and one for KCl solution)*

Falcon tubes (50-mL) and racks to support and store the tubes. *Prepare as many tubes as roots are being sampled for water rinse.*

Liquid Nitrogen

Measuring (also called graduated) cylinders

Pad of 7.5-cm^2Post-its®

These will be used for writing labels, start time, end time, type of ^{15}N and any comments. Old business cards work too, but Post-its stick to the tubes, which helps with tracking.

Paper bags

Paper towels

Pens (Fine tip permanent marker pens, normal pens/pencils)

Phones (cell/mobile phones or watches linked to mobile phones for keeping time)

These are better than unconnected wall clocks because they are already synced.

Plates, 96 well

Razor blades/pruners/fine secateurs

Scales that can accurately measure milligrams (mg) and grams (g)

Spade

Spatulas

Tin capsules, pressed (8 x 5 mm; Elemental microanalysis or equivalent)

Tweezers, long handled (2)

One for the person putting roots in and out of solution, and one for the person taking the photos.

Tweezers, (2) for handling the tin capsules

We *recommend one set round tipped and straight, and the other with a 60 degree bend.*

Weighing boats for weighing granulated/powdered chemicals

METHOD

Plant selection

1. Mark plants for treatment.

Once the plants are at the specific developmental stage to address the research question of interest, it is good to mark the plants using flagging tape the day before treatments, to avoid confusion on the day.

It is very important that the root type and leaf number (V-stage) is reported for any experiment. Each root type behaves differently, and the leaf number can be used as an indication of plant developmental stage.

Labelled solution preparation

Preparing ^{15}N labelled mixtures

2. Determine the number of roots to be sampled.

Ideally, at least 3 roots from each root type from the same plant should be fed each treatment (for example $^{15}NO_3$, $^{15}NH_4^+$, and ^{15}Glycine) simultaneously (or as close as possible).

3. Calculate amount of each stable isotope source required. For instance, to prepare ^{15}N solution for 90 roots, for a 3 mM total N (the exact concentration depends on the research question), comprising of 1 mM of each source of N, and considering 14 mL of solution per root (see note), the amount of each label should be as follows (Table 1):

- For $K^{15}NO_3$ (10% enriched; MW = 101.1 g/mol), 0.131 g of $K^{15}NO_3$ are needed for 1.3 L of milliQ water.
- For $(^{15}NH_4)_2SO_4$ (10% enriched; MW = 101.1 g/mol), 0.086 g of $(^{15}NH_4)_2SO_4$ are needed for 1.3 L of water.

Note that 1 mM N = 0.05 mM of $(NH_4)_2SO_4$

- For ^{15}Glycine (98% enriched), 1 mM = 0.0976 g for 1.3L. But given that we only need 10% enrichment, 0.01 g of ^{15}Glycine + 0.087 g of unlabelled glycine are needed for 1.3 L of water.

In our set up, each root is fed the solution in a 15-mL Falcon tube (we fill to ~14 mL, to allow space for root displacement of solution). To saturate the uptake system, the total N concentration we use is 3 mM total N, comprising of 1 mM of each source of N ($^{15}NO_3^-$, $^{15}NH_4^+$, ^{15}Glycine).

4. Prepare labelled nitrate mixture based on calculates made in previous step. Weigh $K^{15}NO_3$, $(NH_4)_2SO_4$, and glycine (Table 1), add them to the same bottle, add the appropriate amount of deionised water (using a measuring (graduated) cylinder), and mix well.

   Transfer 13-14 mL of the solution into separate 15-mL ml Falcon tubes, enough for all samples. Freeze if not using the same day (we store for up to a week). *The volume here is not as important as having an accurate concentration. Each root will be a different volume and so will displace the solution differently.*

5. Prepare labelled ammonium mixture. To do this, repeat Steps 3-4 but using $(^{15}NH_4)_2SO_4$, KNO_3, and glycine (see Table 1).

6. Prepare labelled glycine mixture. Repeat Steps 3-4, but using ^{15}N-glycine, KNO_3, and $(NH_4)_2SO_4$ (see Table 1). *Note that because we use ^{15}N-glycine with a higher enrichment level than needed, we dilute the enrichment with ^{14}N-glycine – see Step 2 and Table 1 for an example.*

Preparation of other required solutions

Preparation of 10 mM KCl

At the end of the ^{15}N treatments, roots are rinsed with KCl to remove any nitrogen sticking to the root surface and then rinsed in water.

7. Determine quantity of 10 mM KCl solution required (1 tube of solution per root)

   Each root needs to be rinsed with 10 mM KCl but the volume is not critical. We use 15-mL Falcon tubes. For 3 sets of 90 roots = 270 x 15 ml Falcon tubes with 14 ml each of 10 mM KCl (3.78 L total volume) are needed.

8. Prepare enough 10 mM KCl for the required samples. Use deionized water. Distribute ~13-14 ml of the mix into separate 15-mL Falcon tubes.

   These tubes can be stored at room temperature until use (we make them up the day before).

***MilliQ water*:**

9. Fill 50-mL Falcon tubes with 40 mL of milliQ water.

   For the 3 sets of 90 roots, fill 270 x 50-mL Falcon tubes with about 40 mL of milliQ water. These can be stored at room temperature until use (we make them up the day before).

^{15}N feeding procedure for intact roots

We prefer to do all the feeding experiments before midday, to ensure we are measuring uptake during the most active time of day and before plants begin to close stomata to minimise water loss at the hottest time of the day.

Set-up

10. Collect ^{15}N solutions (freshly prepared or from the freezer) and the water and KCl solutions.

11. Collect:

- Extra 15- and 50-mL tube racks
- Square Post-its
- Fine tip marker/pens
- Razor blade/pruners
- Long-handled tweezers (2)
- Paper towels
- Camera, blue background and ruler
- Paper bags for drying roots
- Box to store root samples at the end
- Bucket for rubbish (*with appropriate waste bag for your institution*)

12. Transport everything to the plant area (in our case, for intact sampling, we do this in the glasshouse).

13. On a bench, set up separate areas with:

- ^{15}N solutions (each tube clearly labelled), and nearby a stack of Post-its.
- KCl and water tubes, for rinses.
- Imaging stage

  We use a dark blue plastic folder or the back of a lab book, a ruler on the blue background, and a camera set up at a fixed height above the blue background.

- Paper bags

  For placing each finished root in for oven drying (usually located next to the imaging stage).

Treatment application

14. Tilt boxes backwards and remove the front (ours have clear window panels that can be removed individually) to allow access to the roots.

    You may have a different set up – the point here is to prepare plants for root access.

Be careful not to damage the plants in preparation.

15. Carefully tease roots for nitrogen uptake from the soil.. Leave them attached to the parent plant and be careful not to damage them.

16. Begin treatment. We describe the process for one root:

    i. Take a ^{15}N solution and carefully guide the root into the tube.

    ii. Support the tube by poking it into the soil (Figure 1), ensuring the root remains as far into the tube as feasible (up to 15 cm, which is the length of the tube). Avoid pushing the root so far in it breaks.

    iii. On a Post-it, record each root type and the ^{15}N type that has been fed to the root. Record the exact start time of the treatment (Figure 2).

    iv. After 30 minutes, cut the root at the top of the 15-mL tube (take note of the exact time).

    v. Remove the root from the tube.

    vi. Put it in the corresponding KCl tube and gently invert the tube a few times, to rinse it. *This needs to happen quickly to stop the uptake of stable isotope.*

vii. Write the exact end time of the treatment (as noted in iv) on the Post-it

Aim for 30 minutes, but if it is accurately recorded, it is not a problem if it is 29 minutes or 32 minutes, for example – this information will be accounted for in the downstream calculations.

viii. Remove the root from the KCl and place in the corresponding tube containing water. Invert the tube a few times.

ix. Take the root out of the water, pat it dry, and place it on the imaging stage.

x. Take a photo, including the Post-it with the relevant details (Fig. 2).

xi. Place the root in a paper bag along with the Post-it note associated with the root.

The root is now ready for drying down, which will occur after all the roots have been treated.

xii. When back in the lab, place each paper bag with the roots in a drying oven until the roots have dried.

We set the drying oven at 70°C. This process usually take 48-72 hours.

xiii. Store dried bags with roots at room temperature until processing. When ready, proceed to "Grinding and weighing dried root samples for analysis".

The list of actions under Step 16 describes each step one by one to make the process clear. In reality, however, multiple roots can be at different stages of the process. For example, all three roots (one for each ^{15}N source) can be fairly quickly guided into ^{15}N tubes (step 16 i). The next plant can be prepared while the first three roots are going through the 30-minute treatment.

CAUTION: the easiest place to get mixed up here is with labels – it is very important to keep the Post-it clearly associated with the root being treated. When the

treatment ends, attach the post-it to the KCl tube, then to the water tube, and then into the paper bag at the end.

If there is more than one person available to work on the treatments, the process becomes even more streamlined – one person preparing plants, one person feeding roots (and ending the treatments), one person rinsing, and one person imaging. This all depends on how much space you have in which to work and how many people are available to help.

Procedure for excised roots from field-grown plants

17. Follow steps 1-14 above.
18. Bring spade, hose, and buckets to the field for plant excavation.
19. Proceed with plant excavation:
    i. Using a spade, and at approximately 20-30 cm from the stalk on all four sides of the maize plant, cut the ground as deep as possible.
    ii. Put the spade in and lever the plant from the ground.
    iii. Put the soil-covered root ball in a large bucket of water.
    iv. If needed, use a hose to further clean the soil away from the roots.

    CAUTION: be careful to minimise root damage during this process.

Treatment

This process is very similar to that described under Step 16 – the only difference is that the roots can be cut from the root ball and suspended in the tubes of ^{15}N solution.

20. Begin treatment:

i. Choose undamaged roots of interest from the root ball and cut them as long as possible. *The roots chosen will depend on the research question.*

ii. Put each root tip into a tube containing the relevant ^{15}N solution, ensuring the cut end remains out of the solution.

We sometimes use bulldog clips or pins if they are shorter than the tube so they don't fall in.

iii. Prepare the corresponding Post-it label as described in step 16 iii, listing the plant identification, root type, the ^{15}N source, and the start time.

iv. After 30 minutes, trim the root at the top of the 15-mL tube if longer than the tube

v. Remove the root from the tube.

vi. Put the root in the corresponding tube containing KCl and gently invert the tube a few times.

vii. Write the exact end time of the treatment on the Post-it.

Aim for 30 minutes, but if it is accurately recorded, it will not be a problem (29 or 32 minutes for example – this information will be accounted for in the downstream calculations.

viii. Remove the root from the KCl and place in the corresponding tube containing water. Invert the tube a few times.

ix. Take the root out of the water, pat it dry, and place it on the imaging stage.

x. Take a photo, making sure to include the Post-it (Fig. 2)

xi. Place the root in the paper bag along with the Post-it associated with the root.

The root is now ready for drying down, which will occur after all the roots have been treated.

21. When back in the lab, place each paper bag with the roots in a drying oven until the roots have dried.

We set the drying oven at 70°C. This process usually take 48-72 hours.

22. Store bags with roots at room temperature until processing. When ready, proceed to "Grinding and weighing dried root samples for analysis".

As described in at the end of Step 17 for the rhizobox sampling, this process in the field can also be streamlined. We have found that a minimum team of 4 people is ideal, with one person excavating, one person cutting roots and starting the treatments, one person ending treatments and rinsing, and one person imaging the samples. An extra person is helpful for keeping track of labels, and if we measure anything else at the same time, we add a person to manage that task. Following this protocol, we can process about 15-20 plants between 8-11:30 am.

Grinding and weighing dried root samples for analysis

Once the roots have been dried and left at room temperature, they need to be ground to a powder so they can be analysed for ^{15}N content. To determine the amount of ^{15}N taken up per mg dry weight, you will need to know the amount of tissue that is analysed.

Sample grinding

23. Set up and grind samples:

i. Wash ceramic mortar and pestles with clean water and dry the entire surface with paper towels (*the more you have available, the quicker the process will be*).

ii. Place the mortar and pestles on a clean work surface in the laboratory, along with a small metal spatula.

iii. Stack the paper bags with dried roots to one side of the workspace.

iv. Prepare a place to put the empty bags after the root and label have been removed.

v. Prepare a rack with 1.5-mL Eppendorf tubes and a permanent marker to label the tubes.

vi. Collect liquid nitrogen in an appropriate flask and keep by the work station (following the safety protocols of your institution).

vii. Take the Post-it label from the first sample bag and write the label on the first Eppendorf tube.

viii. Wearing safety glasses, pour some liquid nitrogen into the first mortar.

ix. Take the first root from the bag and place it in the first mortar.

x. Use the mortar and pestle to crush the dried root samples into fine powder.

xi. Use a spatula to fetch all the powdered content in to an appropriately labelled 1.5-Eppendorf tubes and keep erect on racks.

xii. Wash and dry (using paper towels) any used mortar and pestle before crushing the next sample, to avoid contamination.

xiii. Repeat steps vii-xii for the next sample

Sample weighing *We send our samples to the UC Davis Stable Isotope lab. What we describe below, is based on the guidelines provided by the UC Davis Stable Isotope lab (https://stableisotopefacility.ucdavis.edu/), but you may have access to another facility of an in-house analysis suite. Either way, make sure you check with the analytical team in charge for any requirements they have either for training or for sample preparation.*

For Step 25, do not touch the samples or tin capsules with your hands (use tweezers or spatula), and keep your workspace clean to avoid cross contamination.

24. Encapsulation for analysis:

    i. Place a tin capsule on a scale and tare the scale.

    ii. Keeping careful track of labels, place each powdered sample into the tin foil capsule C*heck carefully with the lab that will process your samples for the amount of sample required. In ours, with 10% enrichment, we aim for about 8 mg of each ground sample*.

    iii. Record the exact weight of the sample that has been transferred to the capsule.

    iv. Carefully squeeze the lid of the tin capsule, shut using tweezers, and fold the top over to make a tight seal. *UC Davis Stable Isotope Facility provide an excellent set of images that illustrate this process: https://stableisotopefacility.ucdavis.edu/encapsulation-tips-carbon-and-nitrogen-solids.*

    v. Using the tweezers, gently squash the tin capsule into a ball. Be very careful not to damage the wall of the capsule.

    vi. Transfer the tin ball containing your sample into the well of a 96-well plate and record the well.

    We print a table in the format of a 96 well plate. In each cell, we write the label of the sample that was put in the corresponding well, and the weight of the sample.

    vii. Repeat steps i-vi for all your samples.

    viii. Send the samples for analysis following any other requests from the laboratory running the ^{15}N analysis.

Data processing

25. Convert raw data to uptake rate per unit dry weight (µmol g^{-1}dw).

    For this you'll need to look first at the data provided by the lab and convert that into a total amount of ^{15}N per g (or per mg) dry weight in each sample. That may involve dividing the ^{15}N by the exact weight of each sample if it is not already provided in that format.

26. Convert data to uptake rate per unit dry weight per hour (µmol g^{-1}dw h^{-1}). To do this, divide the amount of ^{15}N uptake by the number of minutes that specific root was fed the stable isotope mix (written on the post-it notes) and then multiply by 60. This will provide the uptake rate per hour.

27. One option for exploring the data is to present the uptake rate relative to root thickness. To do this measure the thickness of each root from the photos taken in Step 16x and Step 20x. Plot the uptake rate by the root thickness.

DISCUSSION

Using the images taken of the roots after the treatments and before drying, the root thickness can be measured and correlations explored between root thickness and nitrogen uptake. The larger the surface area-to-volume ratio (thinner roots), the more epidermal cells and root hairs there will be in the final dried and analysed tissues, such that thinner roots have a higher number of epidermal cells per root gram dry weight compared to a thicker root. As this is where the first point of entry to the root occurs, it is useful to standardize for diameter to see if environmental or genotype differences influence the amount of uptake per unit surface area. In a perfect world, these data can be

compared to transporter expression – however, it is worth noting that there are many different transporter families for each nitrogen source. The data collected using stable isotopes provides information on the net influx regardless of transporters or losses through efflux, and so represent the amount that is available for plant processes.

This protocol has also avoided dictating exactly when roots should be sampled for stable isotope studies. The reason for this is that root emergence and maize growth in general differs between genotypes and in different environmental conditions. Similarly, a single V-stage (Figure 3) may have several days between them and in some circumstances the root growth may change dramatically in this time. For example, the authors see big changes in brace root growth from day-to-day - one day a root may be aerial and not in contact with soil and within 2 days it can have penetrated the soil and be exposed to dramatically different subterranean environmental conditions. The best date for stable isotope uptake also depends on the research question being asked. One study may be interested in comparisons between root types at a particular root age, another may be interested in how uptake changes over time in a single root type. These are both very interesting questions and require the researcher to decide the best timing for stable isotope uptake for their system. The essential point is to clearly explain what timing was chosen, which root types were selected for study (including which whorl number for crown or brace roots), how the decision was made, what v-stage that correlates to and what the plant morphology is at that V-stage in the genotype and environmental conditions in which the study is conducted.

When considering whether to do intact or excised roots, it is important to acknowledge the limitations of each option. For excised roots, the uptake can be overestimated by 30% (Brackin et al. 2015), however the advantage is that this method can be conducted on plants grown in the field. It is very difficult to measure intact stable isotope uptake from plants in the field, so artificial systems where the roots can be accessed are typically used like the rhizoboxes shown in Figure 1. These are vertical boxes with a window down one side and tilted so the roots grow down the window. The boxes can be tilted back and the window removed allowing access to the root systems for intact

772 measurements. A simpler (but one-use) system is thin pots that can be cut to reveal the roots, or
773 even simpler (but more disruptive) growing plants in pots and then removing them completely but
774 keeping shoots and roots connected. These systems allow intact measurements but of course are not
775 field-grown plants and have the limitation of restricted growing space. There is currently no perfect
776 system to overcome all the limitations, but the more studies that report carefully on their
777 experimental system, the better the information that will be available when developing new
778 techniques and strategies in the future.

779

780 **COMPETING INTEREST STATEMENT**

781 The authors have no competing interests to report.

782

783 **ACKNOWLEDGEMENTS**

784 Authors wish to thank the Royal Society International Exchange grant IES\R1\211169

785

786 **AUTHOR CONTRIBUTIONS**

787 Conceptualisation – AR; Writing - Initial draft – FDI; writing - Review and editing (AR and FDI).

788

789 **REFERENCES**

790 Brackin R, Näsholm T, Robinson N, Guillou S, Vinall K, Lakshmanan P, Schmidt S, Inselsbacher E. 2015.
791 Nitrogen fluxes at the root-soil interface show a mismatch of nitrogen fertilizer supply and
792 sugarcane root uptake capacity. *Scientific reports* **5**: 15727-15727.

793 Hochholdinger F, Marcon C, Baldauf JA, Yu P, Frey FP. 2018a. Proteomics of maize root development.
794 *Frontiers in plant science* **9**: 143-143.

795 Hochholdinger F, Park WJ, Sauer M, Woll K. 2004. From weeds to crops: genetic analysis of root
796 development in cereals. *Trends in plant science* **9**: 42-48.
797 Hochholdinger F, Yu P, Marcon C. 2018b. Genetic Control of Root System Development in Maize.
798 *Trends in Plant Science* **23**: 79-88.
799 Hostetler AN, Khangura RS, Dilkes BP, Sparks EE. 2021. Bracing for sustainable agriculture: the
800 development and function of brace roots in members of Poaceae. *Current opinion in plant*
801 *biology* **59**: 101985-101985.
802 Saengwilai P, Tian X, Lynch JP. 2014. Low Crown Root Number Enhances Nitrogen Acquisition from
803 Low-Nitrogen Soils in Maize. *Plant physiology (Bethesda)* **166**: 581-589.
804 Yu P, Eggert K, von Wirén N, Li C, Hochholdinger F. 2015. Cell Type-Specific Gene Expression Analyses
805 by RNA Sequencing Reveal Local High Nitrate-Triggered Lateral Root Initiation in Shoot-Borne
806 Roots of Maize by Modulating Auxin-Related Cell Cycle Regulation. *Plant physiology*
807 *(Bethesda)* **169**: 690-704.
808 Yu P, Wang C, Baldauf JA, Tai H, Gutjahr C, Hochholdinger F, Li C. 2018. Root type and soil phosphate
809 determine the taxonomic landscape of colonizing fungi and the transcriptome of field-grown
810 maize roots. *The New phytologist* **217**: 1240-1253.

811

812

813 **FIGURES:**

814 **Figure 1. Maize rhizobox system for studying nitrogen uptake on intact plants.** Boxes are
815 tipped back, opened, and then roots are accessible from the surface. The tubes of labelled
816 solutions are placed on the soil, with the intact root tips within the tube.

817

Figure 2. Root image after stable isotope feeding, rinsing, and patting dry, including Post-it label. Post-it labels include plant and root identifier, source of labelled nitrogen (in the picture the ^{15}N was in glycine (Gly), the start time of the labelled treatments, and the end time of the labelled treatment. Images are taken on a blue background for contrast with white roots, and a ruler is always included in the image for scale calculations.

Figure 3. Vegetative developmental stages in maize. Developmental stages of maize are described based on leaf number. When two leaves have emerged it is called V2 (vegetative, 2 leaves emerged), V6 stage is vegetative with 6 leaves emerged and so on. When tassels form at the top of the plant it is called VT. After this there are a range of reproductive stages based on stages of grain development. It is important to note that different genotypes will produce different maximum leaf numbers. For example, the diagram here reaches VT after V14 but it can be higher. Similarly root development can vary between genotype and environmental condition. For all these reasons it is very important to report the morphology of the genotypes being studied and the stage at which stable isotope uptake is measured. Finally the diagram is not scaled for root-to-shoot relative sizes. The roots are most often at least as deep as the shoots are tall (in field-grown plants).

844

845

846

847

848

849

850 **TABLES**

851 **Table 1: Weights for a sample assay involving 90 plants**. For each molecule, we list the amount per

852 mix that should be added of each N source. Three separate mixes are prepared, to test for the uptake

853 of each of the labelled molecules in separate assays. The amount listed are for a 3 mM total N in

854 each mix, comprising of 1 mM of each source of N, and considering 14 mL of solution per sample to

855 test. Bold means it has ^{15}N – check carefully when preparing.

Label	Amount of KNO_3	Amount of $(NH_4)_2SO_4$	Amount of Glycine	Total volume
$^{15}NO_3$	**0.131 g**	0.086 g	0.098 g	1.3 L
$^{15}NH_4$	0.131 g	**0.086 g**	0.098 g	1.3 L
^{15}Glycine	0.131 g	0.086 g	**0.01 g** + 0.09 g	1.3 L

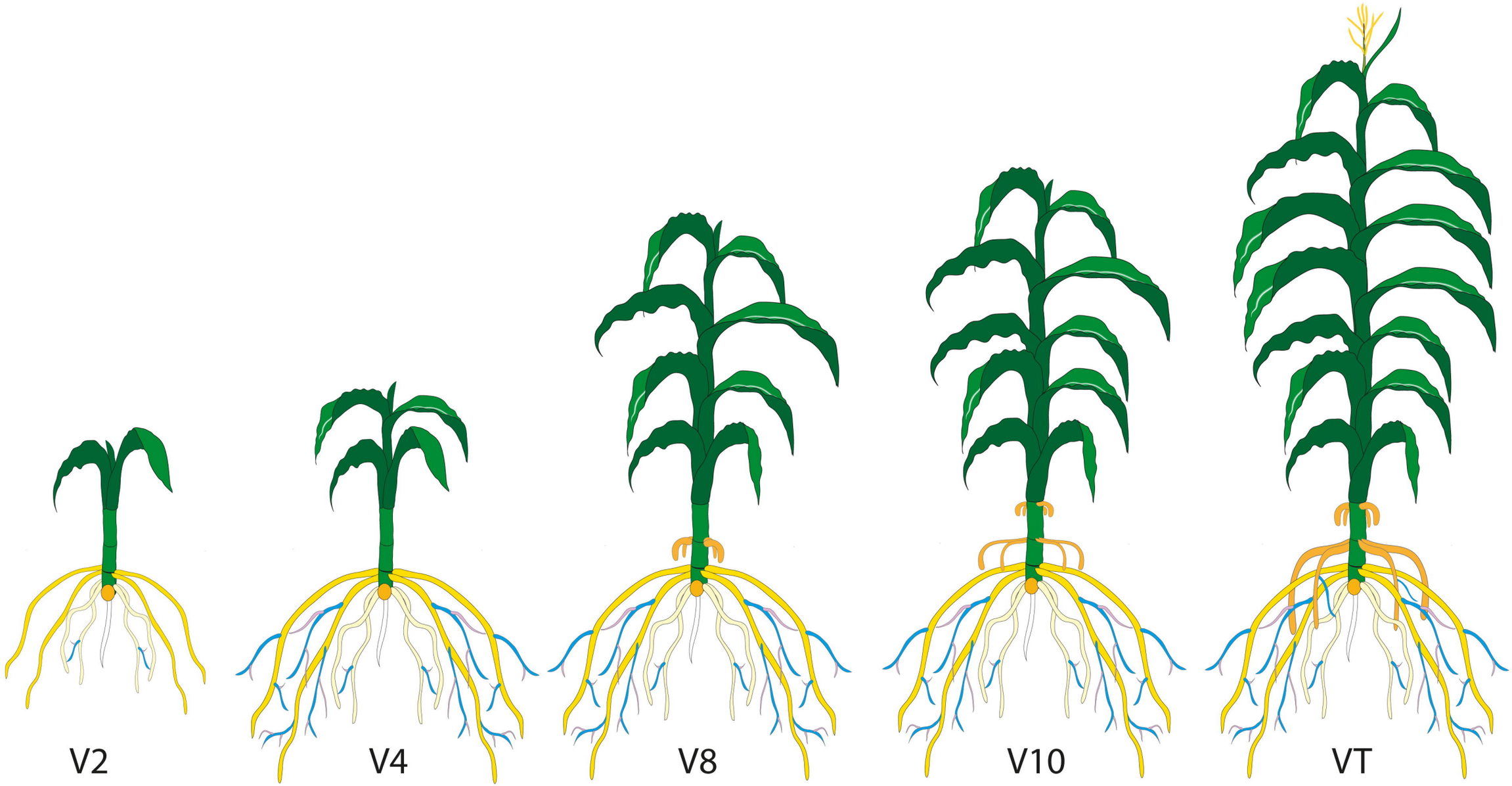
856

857

1358-2-B GG

Start 8:34

End 9:04


V2
V4
V8
V10
VT