Supplementary Information

Intensive field phenotyping of maize (*Zea mays* L.) root crowns identifies phenes and phene integration associated with plant growth and nitrogen acquisition. LM York and JP Lynch. 2015.

Figure S1. Measurements were made with computer assistance using RSAJ, which is available to use with ImageJ. Blue annotations mark root crown measurements, and include stem width (top horizontal line), system width (bottom horizontal line), and the distance between stem and system width (vertical line) for calculating angle with trigonometry, and blue squares count nodal roots, which were counted twice assuming symmetry of the root system. Red annotations mark representative nodal root measurements, including nodal root diameter (top horizontal line), distance to branching (top vertical line), distance along nodal root for counting laterals (middle vertical line), red squares for counting lateral roots, and 3 poly-lines for measuring lateral root length.

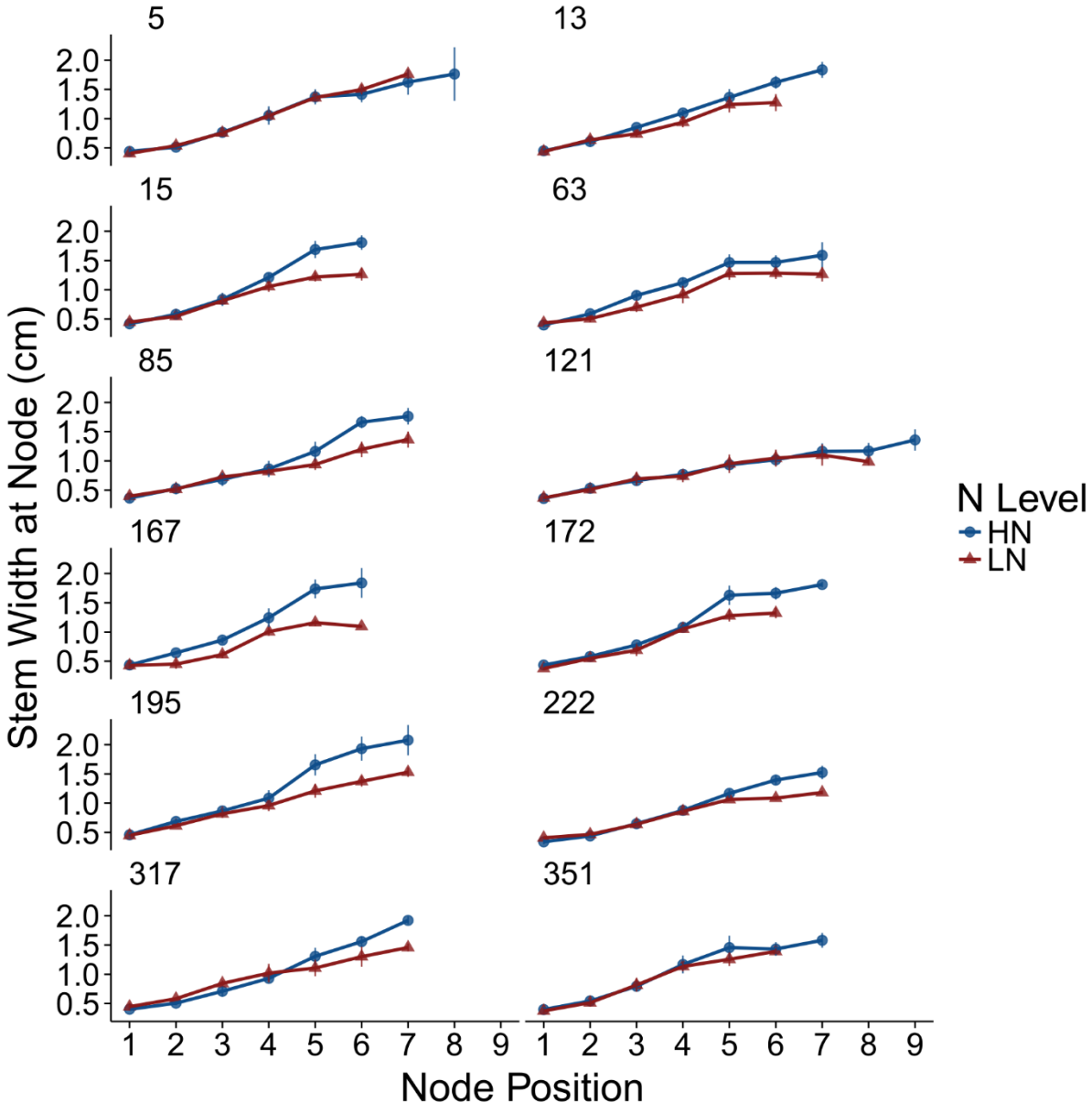

Figure S2. Stem width was measured at each node position in South Africa, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, stem width in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

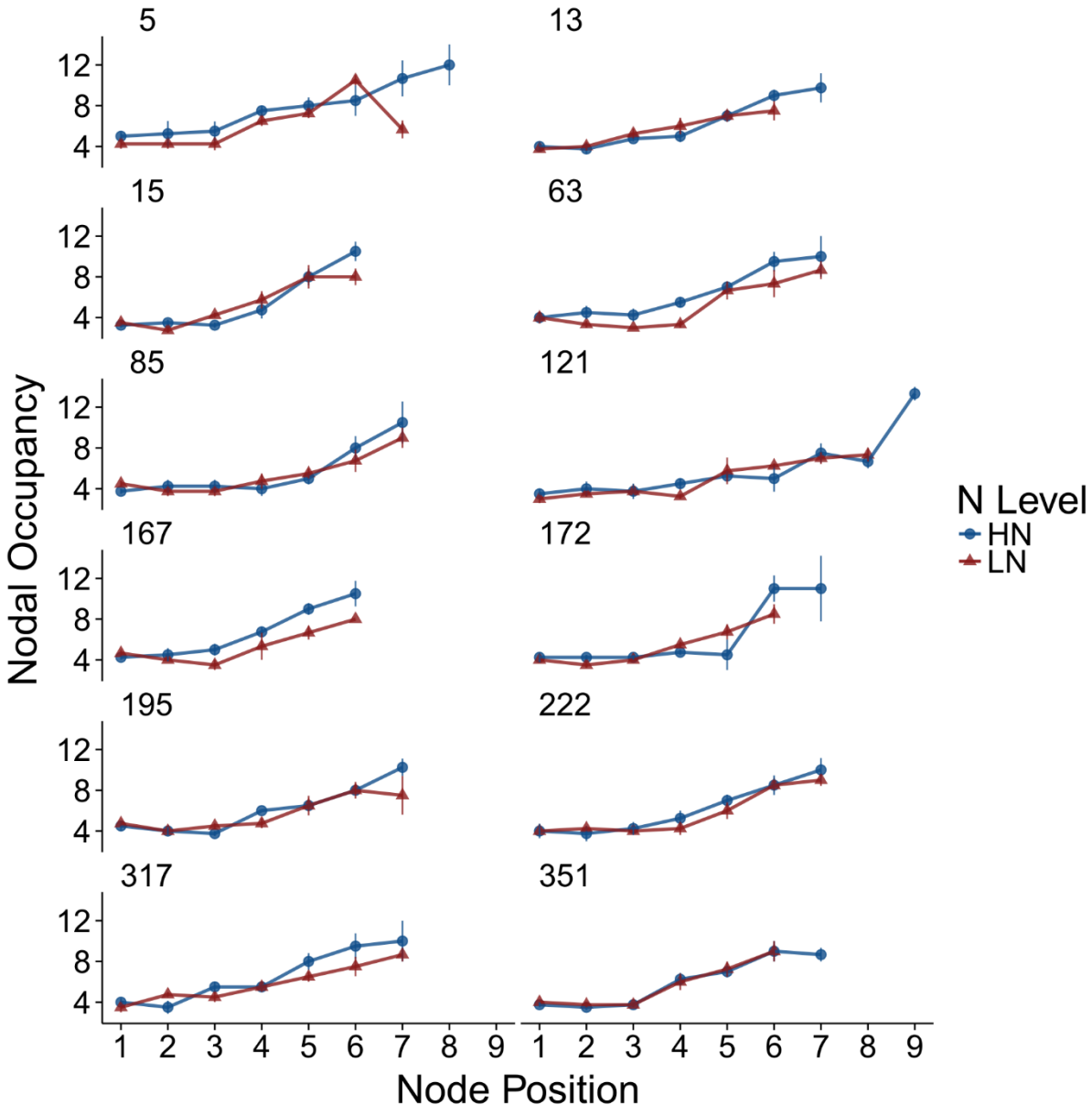

Figure S3. Nodal occupancy, or the number of roots in a node in South Africa, was measured at each node position, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, nodal occupancy in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

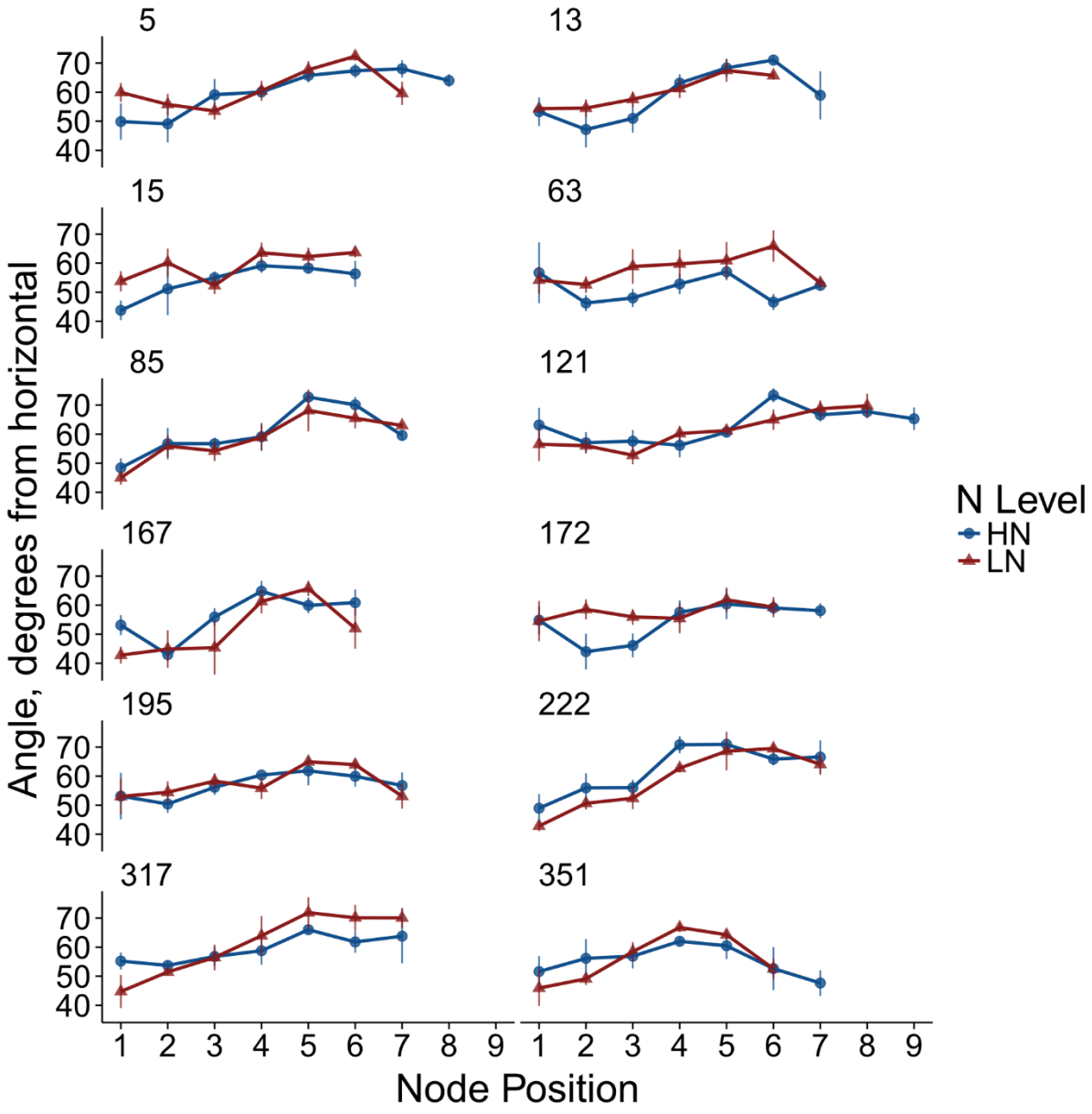

Figure S4. Nodal root growth angle (NRGA) was measured at each node position in South Africa, with position 1 being the oldest whorl. Lower values of NRGA indicate more shallow angles. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, NRGA in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

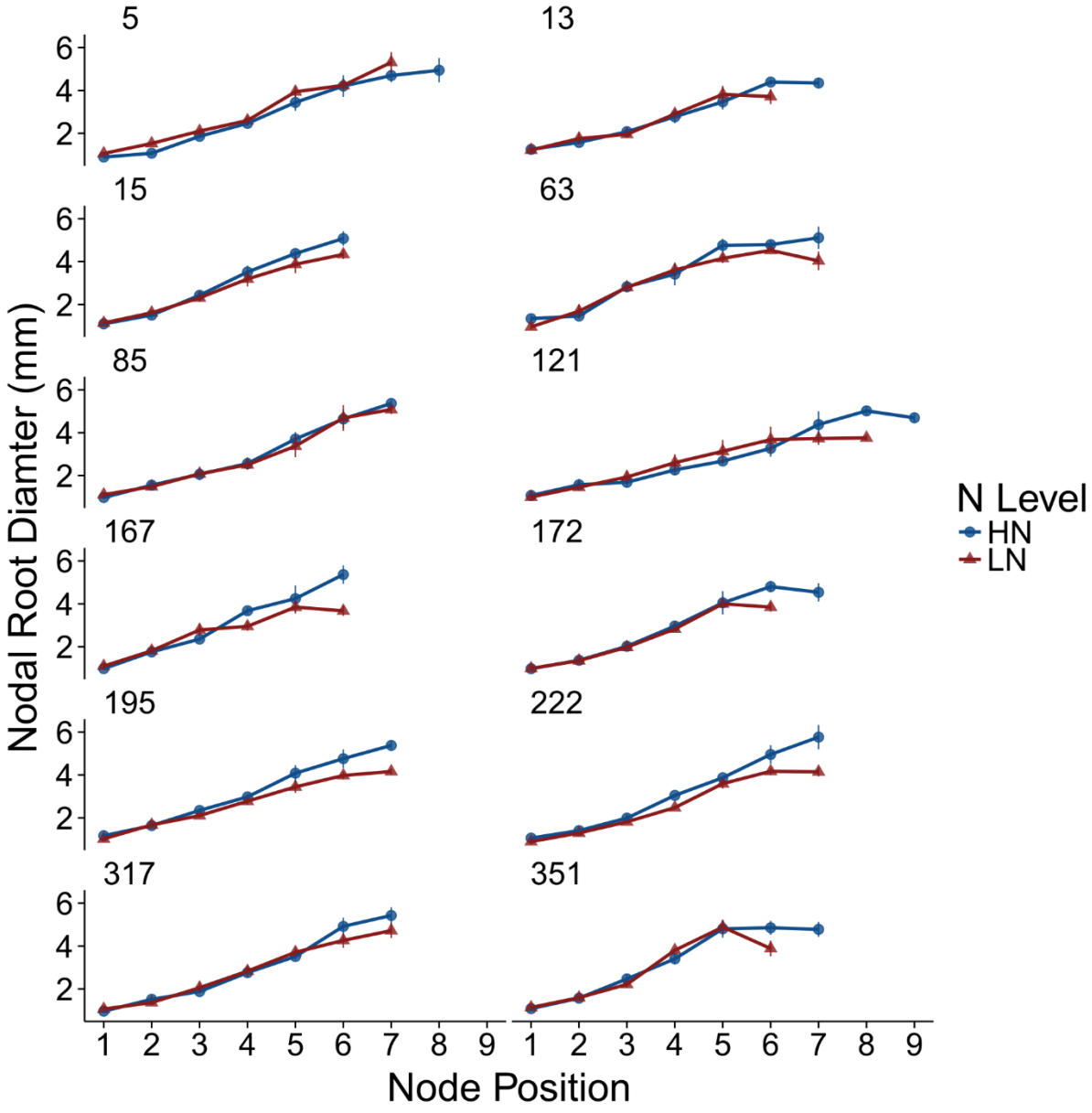

Figure S5. Nodal root diameter was measured on a representative nodal root from each node position in South Africa, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, nodal root diameter in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

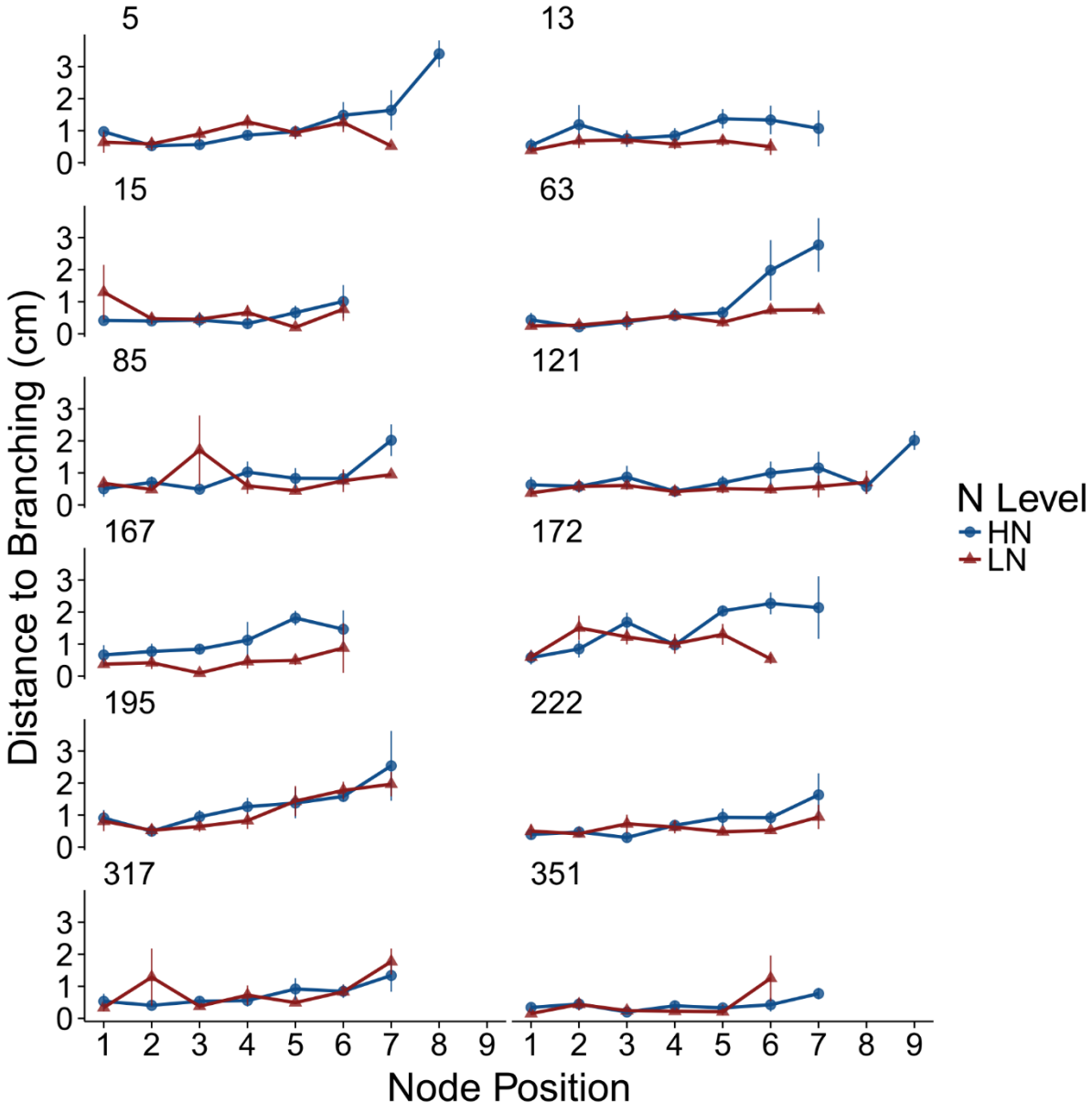

Figure S6. The distance to branching (from nodal root base to first lateral emergence) was measured on a representative nodal root from each node position in South Africa, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, distance to branching in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

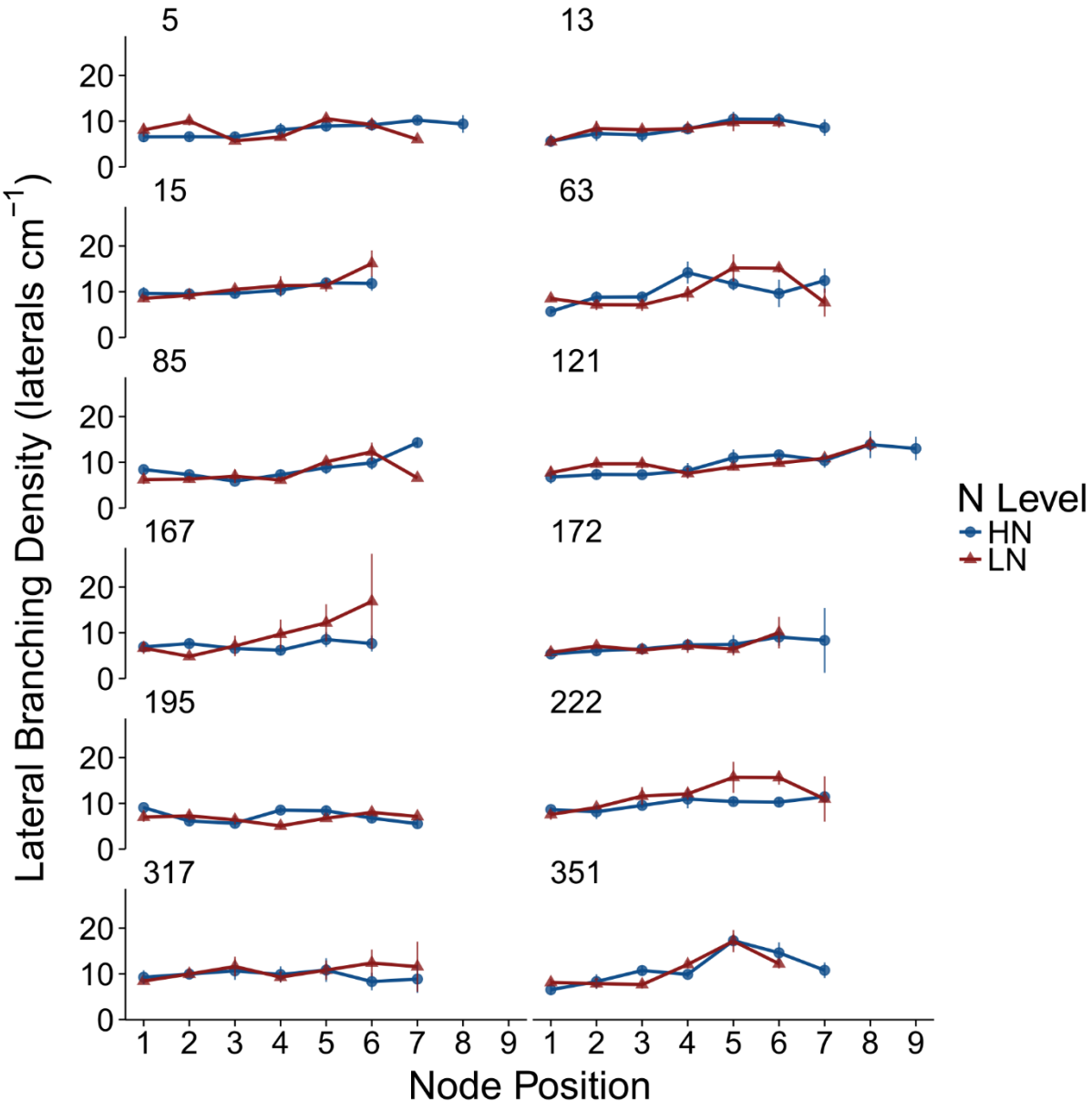

Figure S7. Lateral branching density of a representative nodal root was measured from each node position in South Africa, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, lateral branching density in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

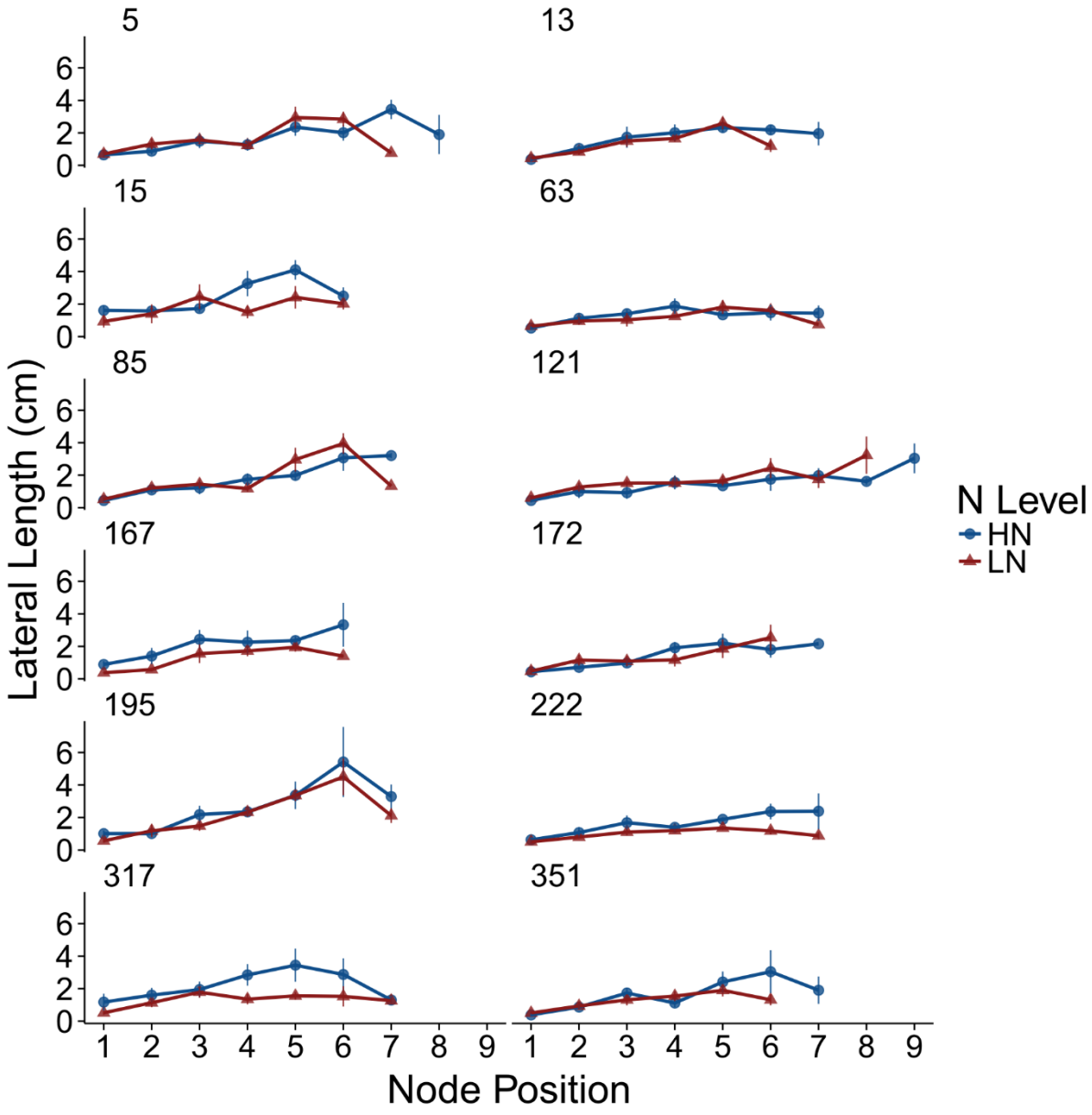

Figure S8. Lateral length (average of 3 laterals per representative nodal root) was measured on a representative nodal root from each node position in South Africa, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, lateral length in high nitrogen (HN) is depicted with closed circles and in low nitrogen (LN) with closed triangles. The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

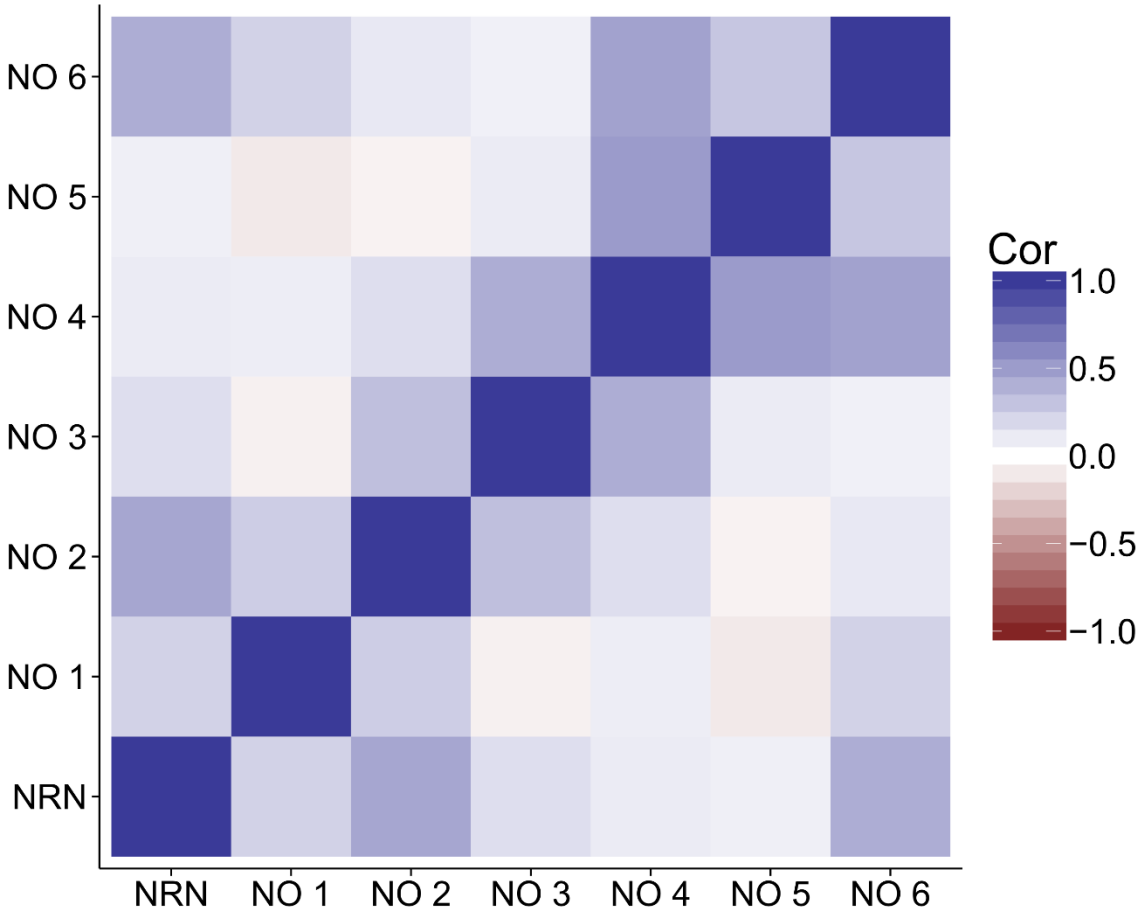

Figure S9. Spearman correlations among the nodal occupancies (NO) of different node positions and total nodal root number (NRN) in low nitrogen soil at URBC in 2014. The numbers following NO indicate the node position, with 1 being the oldest.

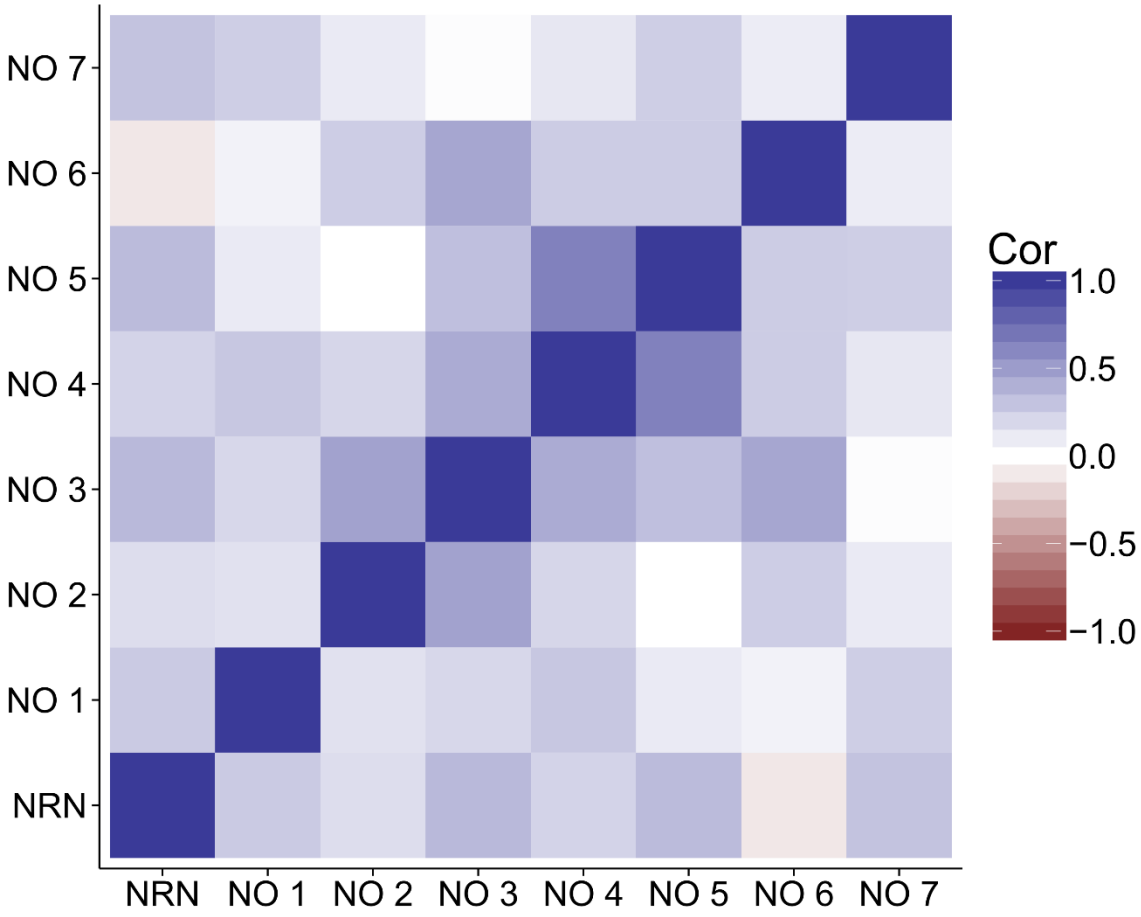

Figure S10. Spearman correlations among the nodal occupancies (NO) of different node positions and total nodal root number (NRN) in high nitrogen soil at URBC in 2014. The numbers following NO indicate the node position, with 1 being the oldest.

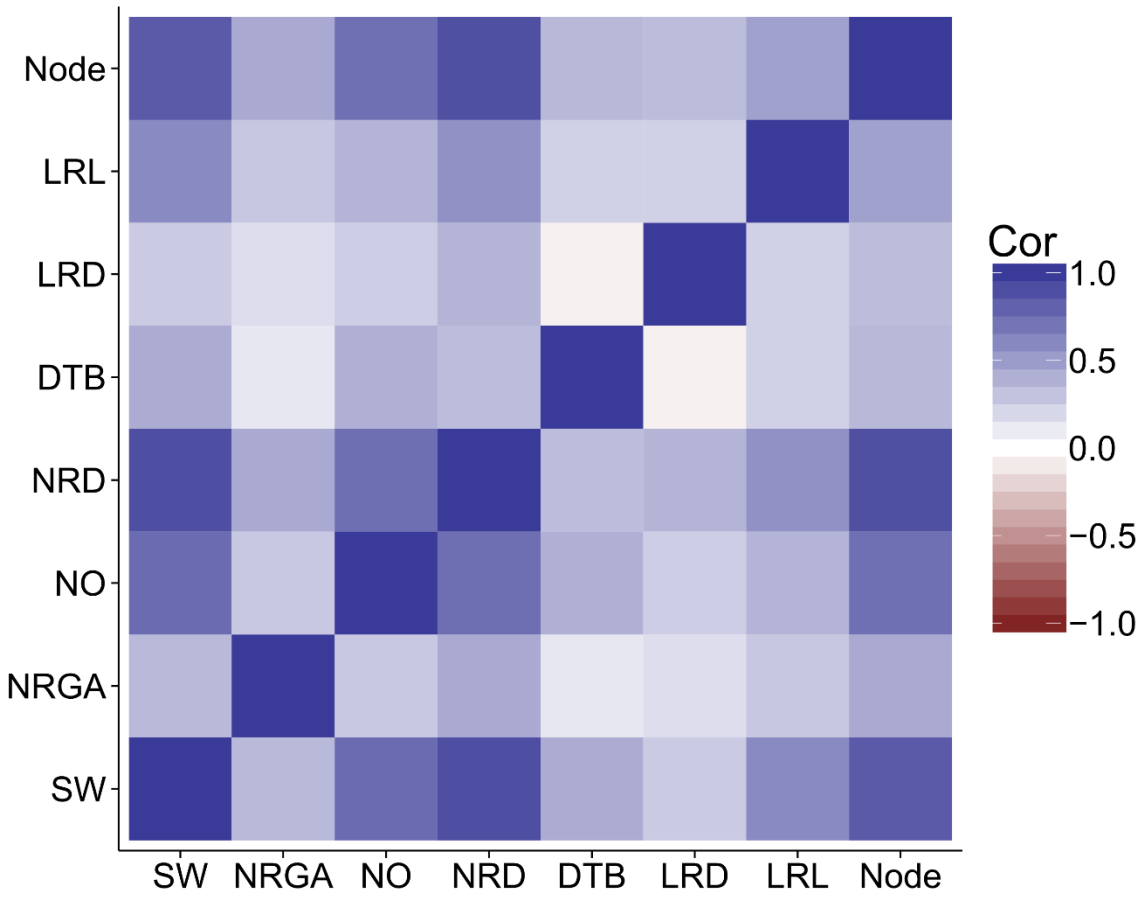

Figure S11. Spearman correlations among all root phenes in all nodes and the node position (Node) at URBC in 2014. Abbreviations are is in Table 1.

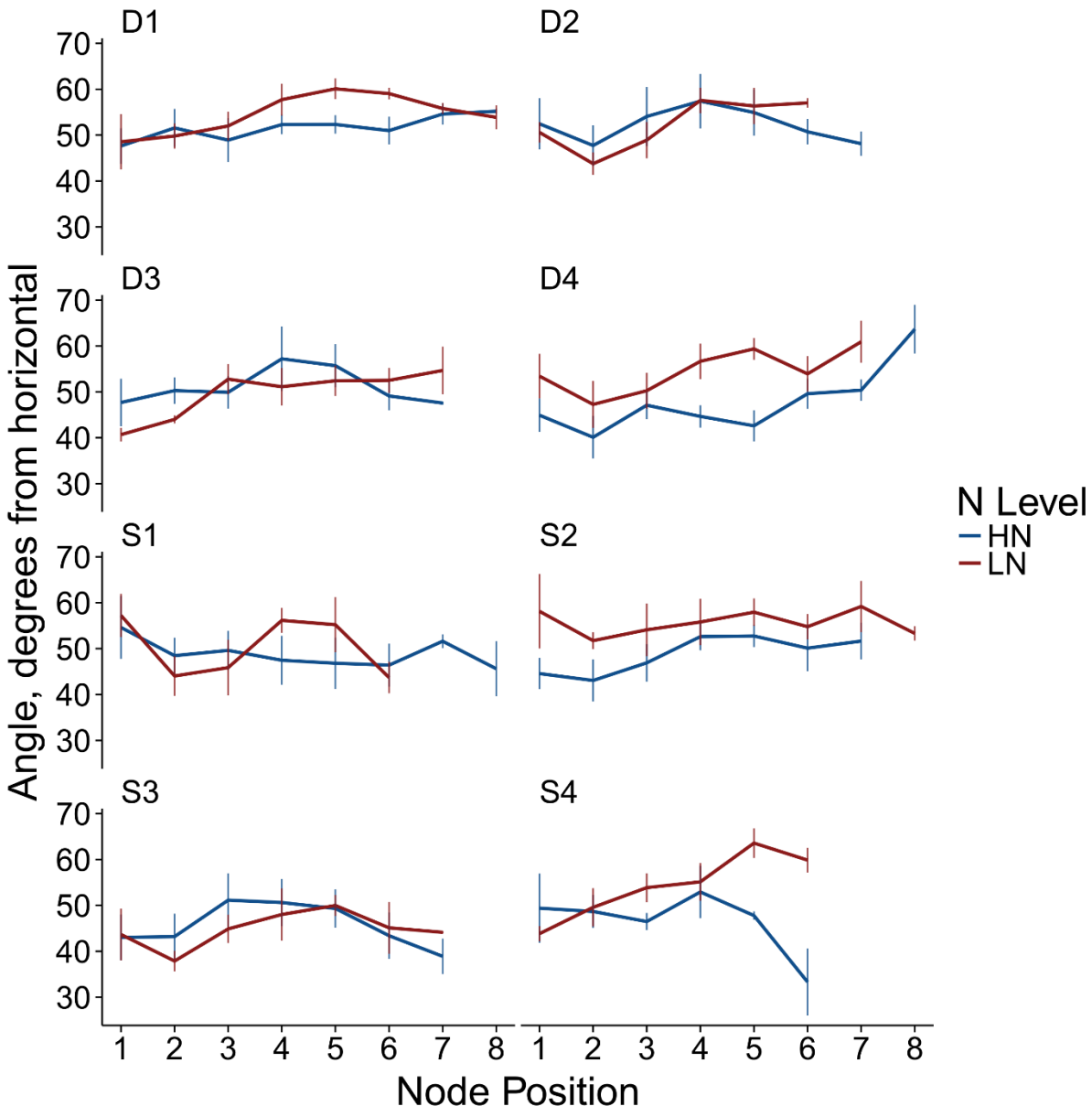

Figure S12. Nodal root growth angle (NRGA) in the USA in 2012 was measured at each node position, with position 1 being the oldest whorl. Lower values of NRGA indicate more shallow angles. 8 panels are annotated with the identity of the IBM maize genotype. Within each panel, NRGA at each node position is colored blue for high nitrogen (HN) and red for low nitrogen (LN). The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

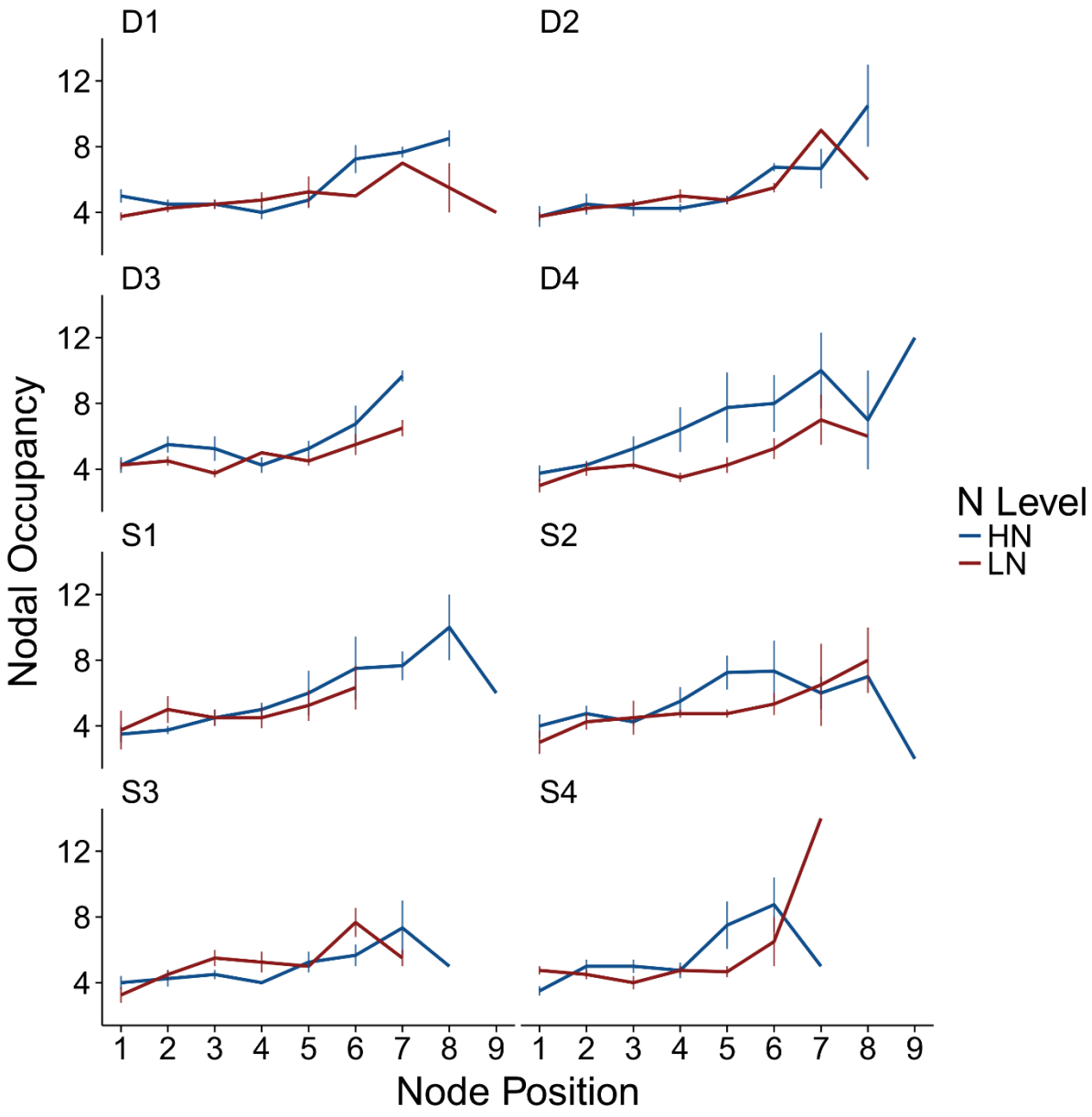

Figure S13. Nodal occupancy, or the number of roots in a node, in the USA in 2012 was measured at each node position, with position 1 being the oldest whorl. 8 panels are annotated with the identity of the IBM maize genotype. Within each panel, nodal occupancy at each node position is colored blue for high nitrogen (HN) and red for low nitrogen (LN). The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

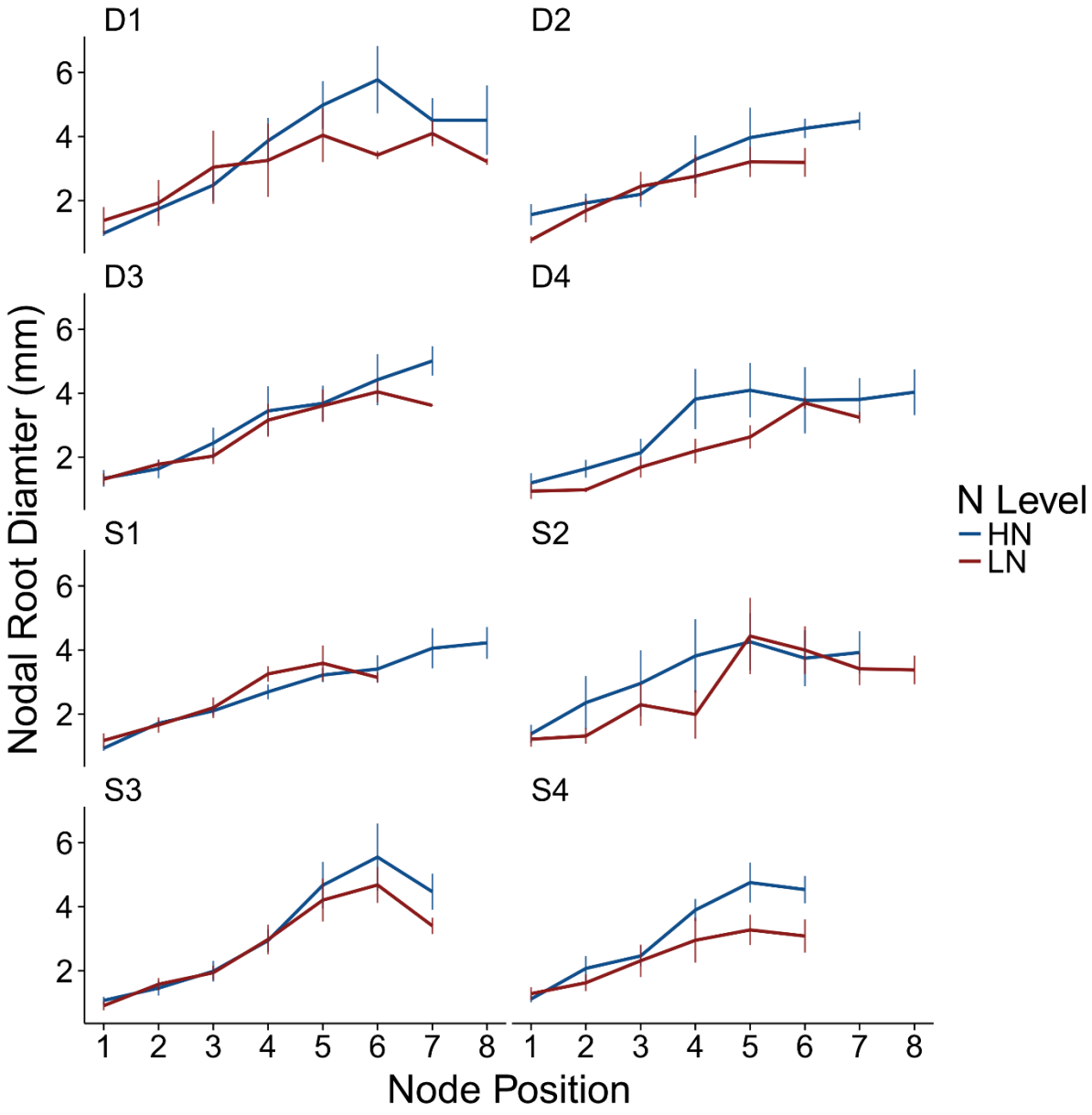

Figure S14. Nodal root diameter in the USA in 2012 was measured at each node position, with position 1 being the oldest whorl. 8 panels are annotated with the identity of the IBM maize genotype. Within each panel, nodal root diameter at each node position is colored blue for high nitrogen (HN) and red for low nitrogen (LN). The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

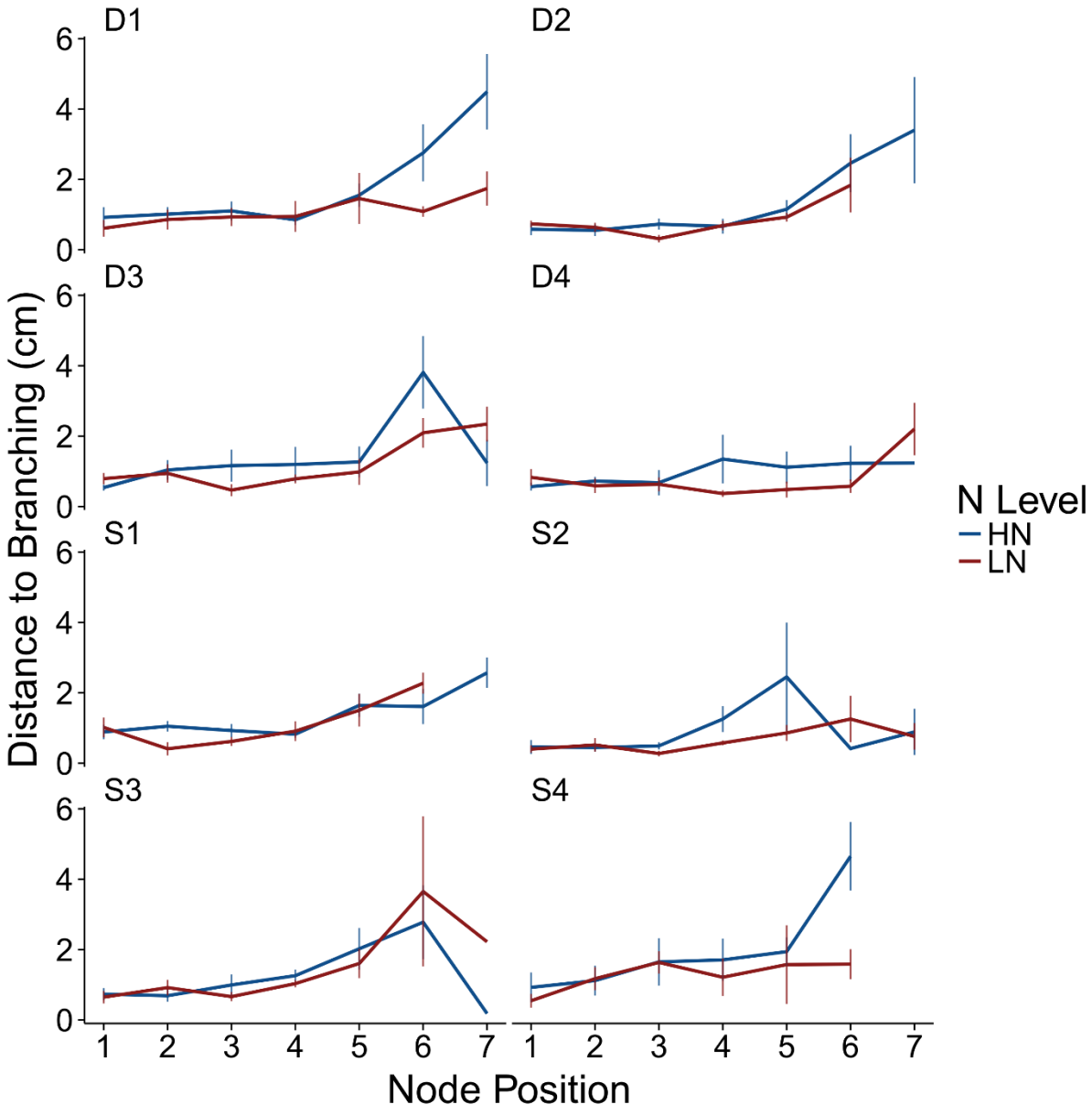

Figure S15. The distance to branching (from nodal root base to first lateral emergence) in the USA in 2012 was measured at each node position, with position 1 being the oldest whorl. 8 panels are annotated with the identity of the IBM maize genotype. Within each panel, distance to branching at each node position is colored blue for high nitrogen (HN) and red for low nitrogen (LN). The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

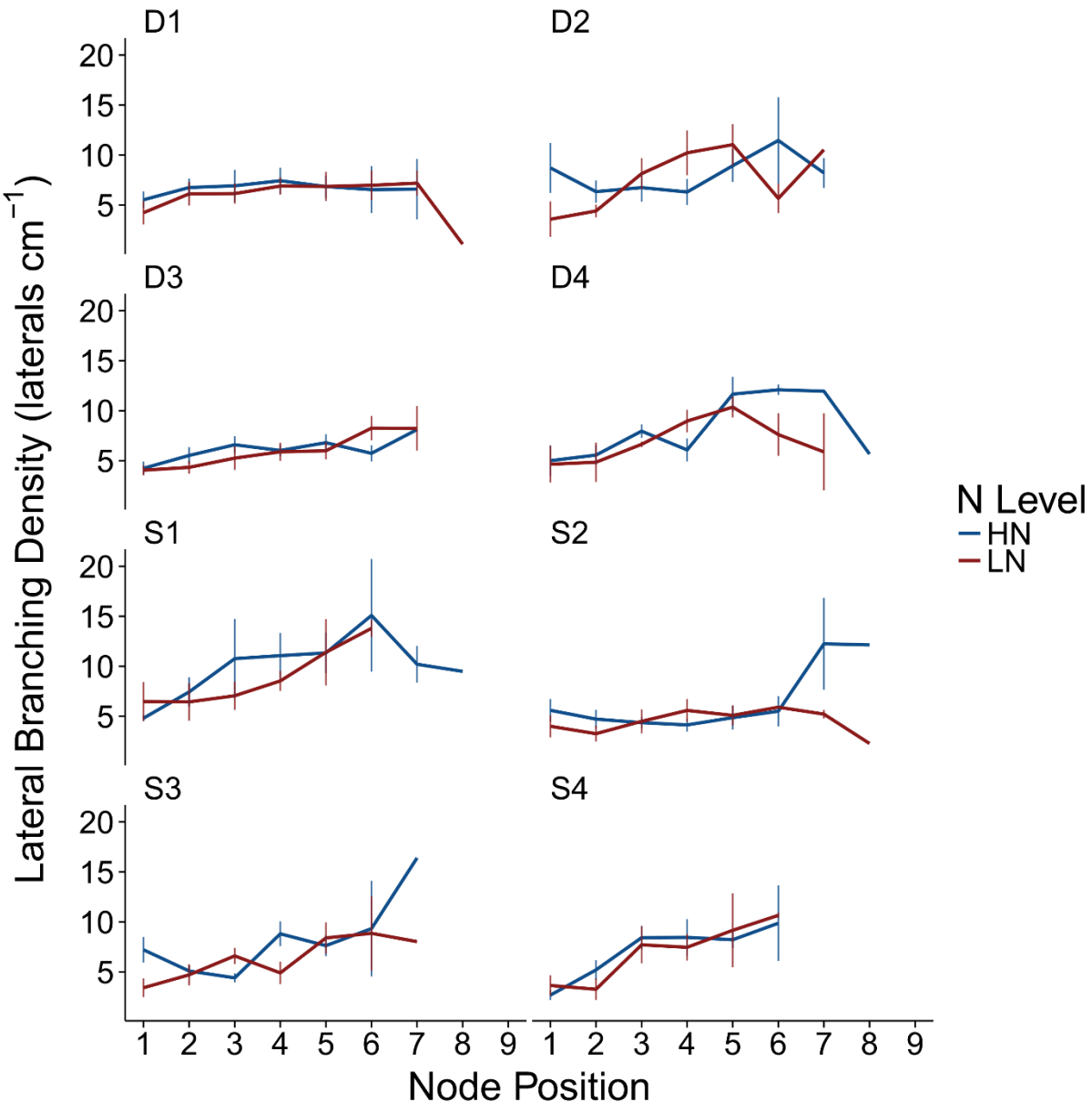

Figure S16. Lateral branching density in the USA in 2012 was measured at each node position, with position 1 being the oldest whorl. 12 panels are annotated with the identity of the IBM maize genotype. Within each panel, lateral branching density at each node position is colored blue for high nitrogen (HN) and red for low nitrogen (LN). The line height at each node position is the mean of 4 replicates, while vertical lines represent standard error.

RSAJ Manual

Compiled by Larry M York, April 4, 2014

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Summary

RSAJ is a project for the ObjectJ plugin for ImageJ. RSAJ was developed for the measurement of root system architecture traits in maize but the ideas can be extended to other species. Traits measured are intended to be the same or improvements as those measured in shovelomics. The objects can be modified to a user's specific situation. RSAJ allows the user to place markers on images, and to edit those markers later. The makers define objects such as points, lines, and angles. The user can review a project any time after completion to check the accuracy of measurements by seeing where the markers are located overlaid on images.

Image requirements

Images can be taken with any type of camera mounted above the root crown. Higher quality data will be generated from higher quality pictures in general. A matte, black background works well, such as a table painted with blackboard paint. A washed root crown should be placed on the background, with one nodal root cut off from that whorl and placed on the side of the image. Keep the stem of the root crown as vertical as possible. Including a scale of a known size is necessary to calculate pixels per length. Best practice includes keeping a tag with the sample identity in the picture as well, such as printed labels. Renaming image files to the sample ID is most convenient, as RSAJ will include the image name in the results. For maize, it is best to at least image the outside brace roots that penetrate the soil along with the most outside crown roots. Brace roots will be pigmented while crown roots will not be pigmented. Simply cut off a whorl of brace roots to reveal crown roots.

Opening RSAJ the first time and adding images to analyze

1. Install ImageJ using their directions and review online documentation

http://imagej.nih.gov/ij/

2. Install ObjectJ using their directions and review online documentation

http://simon.bio.uva.nl/objectj/

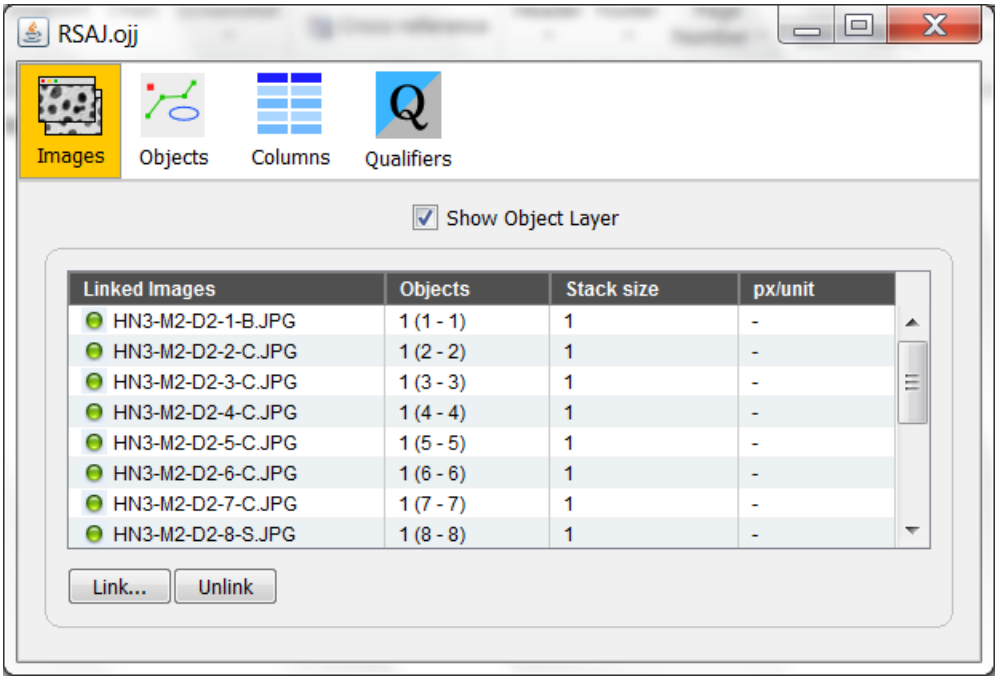
3. Download RSAJ ObjectJ package file to folder containing images

4. Open ImageJ, navigate to Plugins – jars – ObjectJ

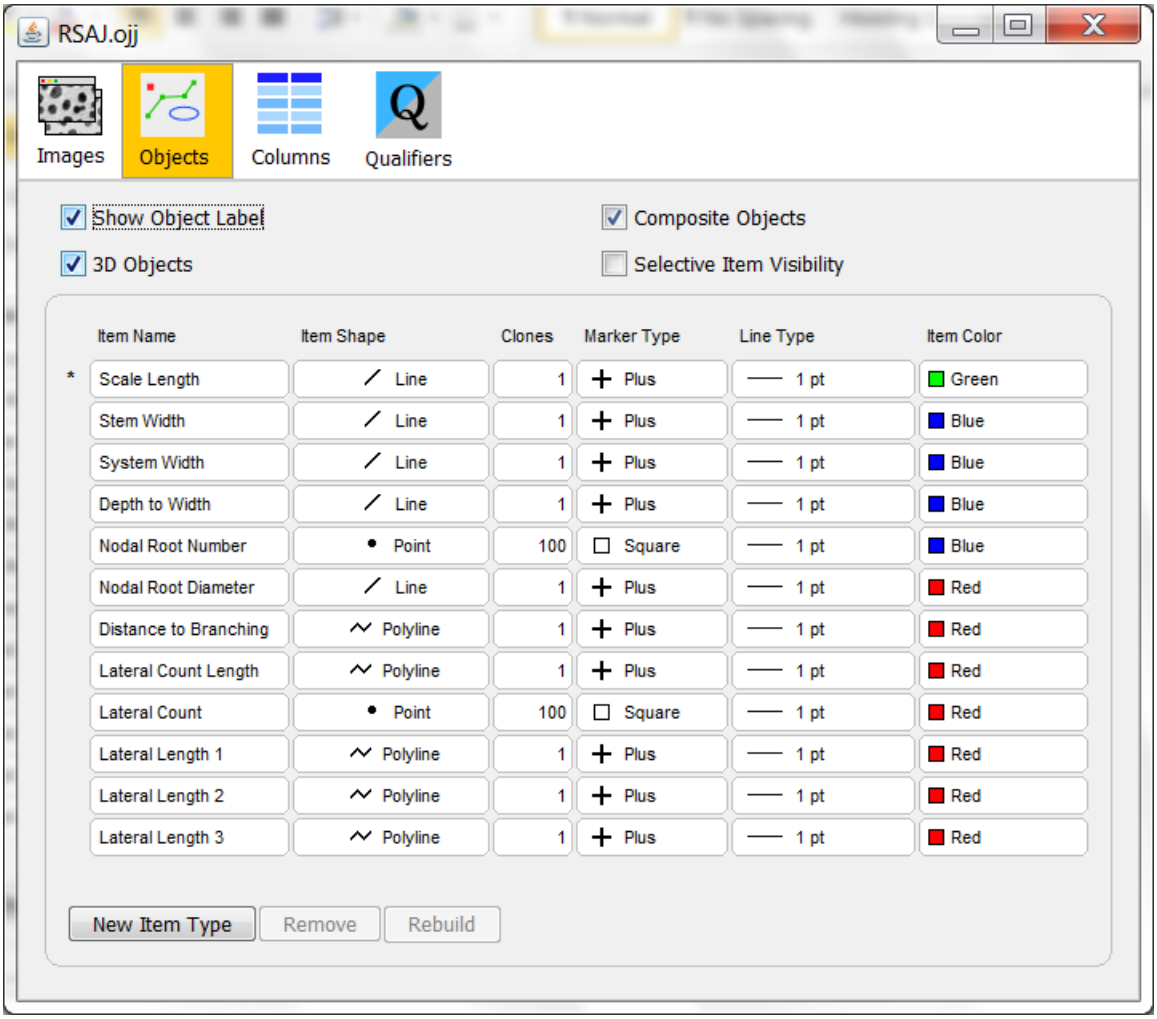
5. Open RSAJ by ObjectJ – Project – Open Project, navigate to folder

6. Drag and drop images intended for analysis into the Images tab of the Project Window (note these images must be in the same directory as the RSAJ project file).

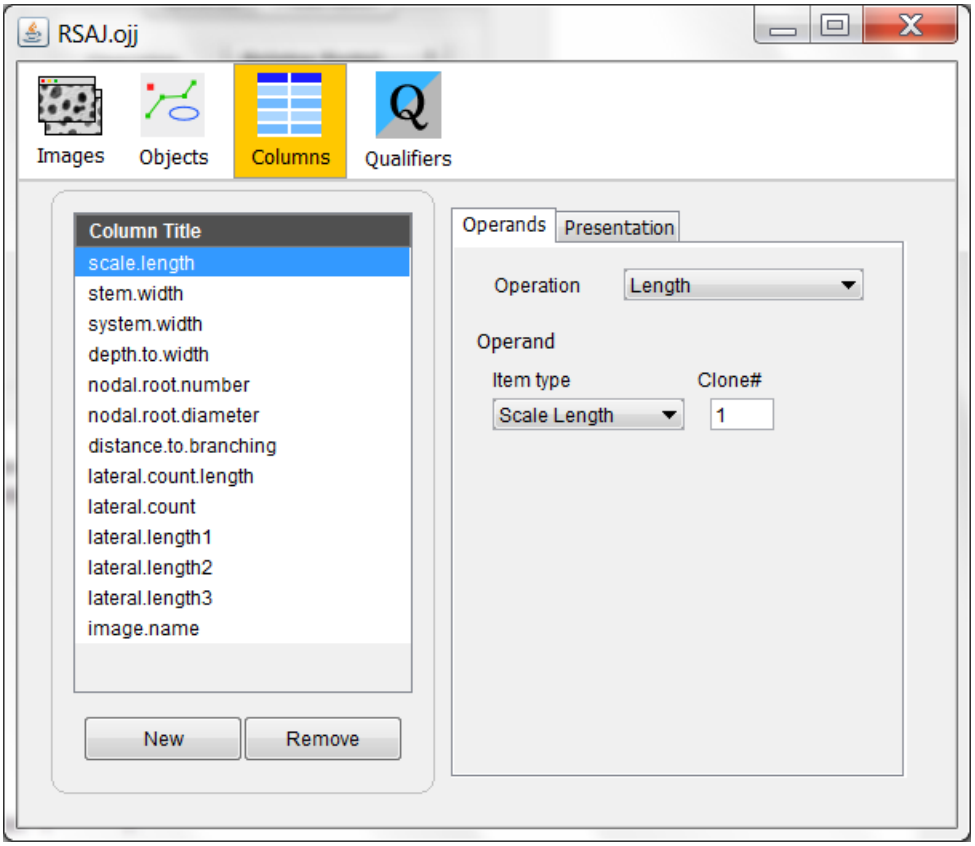
7. Before beginning, become familiar with the Project Window by reviewing the following pages.



The Project Window consists of tabs for Images, Objects, Columns, and Qualifiers. We will not use the Qualifiers tab. The above screenshot shows the Images tab after images to be analyzed have been dragged and dropped inside. Alternatively, the Link button can be used. The images must be in the same folder as the project file.



The Objects tab of the RSAJ project window shows all the traits that will be measured. The scale length is in green, root crown traits are in blue, and traits measured on an individual nodal root are in red. Clones show the numbers for each object, and for most we are only measuring one. Notice that the traits we count (nodal root number and lateral root count) have 100 clones. The clones for counting are just set high enough so you will never exhaust the possible number in one image, your data will only show the number you count. If you don't wish to measure a trait, just remove it from this list. However, nothing needs to be changed to use RSAJ as is. Traits are explained in the protocol, but the names are meant to be self explanatory.



The Columns tab is where objects are interpreted as data via the Operation setting. These operations are used to output your objects to the results. The names of the columns in the data are similar to the names used on the Objects tabs and readily readable by R. Studying this will allow you to make your own length, count, and angle objects as needed. If you don't wish to measure a trait, just remove it from this list. However, nothing needs to be changed to use RSAJ as is.

Protocol for measuring root system architecture in maize using RSAJ

1. Double click on the first image to analyze

2. Measure the length of your scale. Click on one end, then the other to form a line. If the camera zoom and height is constant then you only need to do this for one image. However, it might be safe to always do it.

3. Automatically jumps to measure width of stem where the current whorl of nodal roots originates. Click on one edge of the stem, then the other to form a line. Try to measure exactly where the nodal roots farthest to the left and right intersects the stem on both sides and stay as perpendicular to the growth direction of the stem as possible. Review angle writeup.

4. Automatically jumps to measure root system width. Click on the left most nodal root then the right most nodal root to form a line. Measure the width as far towards the broken ends of the nodal roots as possible while keeping the line perpendicular to the growth direction of the stem and parallel to the stem width.

5. Automatically jumps to measure depth to width, or the height. Click in the center of the stem width line, then in the center of the root system width line, but make sure this line is parallel to the stem and perpendicular to the two widths.

6. Automatically jumps to count the nodal roots. Click on each visible nodal root in that whorl. Because the maize root system is symmetrical, the user can click on each nodal root again to estimate the total number of roots in that whorl. Zoom in before going to 7.

7. Press tab to advance to measures of the excised representative nodal root, starting with its diameter. Click on its left side then right side to form a line near its end that connected to the stem.

8. Automatically jumps to measure distance to branching. This is a polyline, which means each click yields a line segment, and length will be reported by summing the lengths of those segments. This allows us to better follow curves that may exist. First click on the end that was attached to the stem then add line segments by clicking while following the curve of the root. End the polyline where roots begin to emerge.

9. Press tab to advance to measuring the length over which you will count lateral roots. This is a polyline so you can follow the curve of the root over 2-4 cm.

10. Press tab to advance to lateral root counting. Click on each lateral root visible and originating from the area of root that you marked the length over which to count the laterals.

11. Press tab to measure lateral root length. Chose representative laterals and draw a polyline, and press tab to advance to the next lateral.

12. Close the image and start over from step 1 with the next image.

Tricks of the trade and hotkeys

Zoom in and out with the + and – keys. Press and hold spacebar to then click and drag the picture. With ObjectJ, it seems better to zoom in before advancing to the next measurement because it sometimes interprets zooming when on a new item that hasn't been measured as forming a new object. If this happens, then when you start measuring the next trait you will see another number form. If this happens, choose the finger tool from the ObjectJ tools and click on the last thing you measured to go back to editing that object then advance to the next trait. If you make a mistake here, all the data will be kept, and you will simply have to edit the dataset to move the data to the same row.

If you add an extra line segment to a polyline or an extra point when counting, use the backspace key to delete it before advancing to the next trait.

If you need to move a point after placing it, choose the move tool then hold down alt and click and drag the point you wish to move.

If you feel like you need to start over for an image, use the gun tool to delete the whole object.

Sometimes you might find a window missing, you can always bring them up from the ObjectJ menu in ImageJ, but might find these helpful:

Ctrl + shift + F1 = Project Window

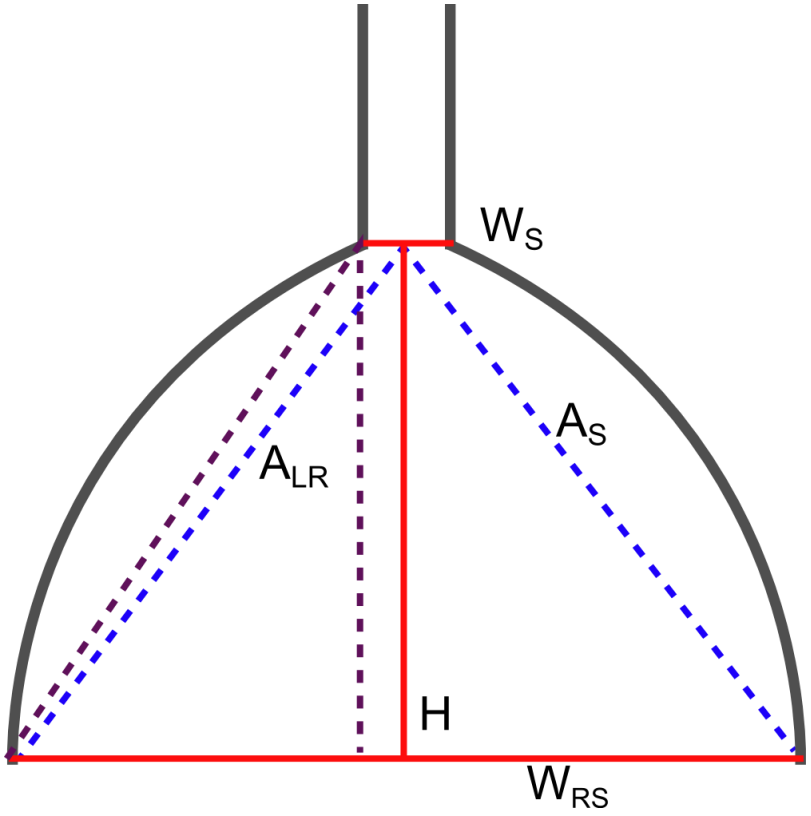
Ctrl + shift + F2 = Tools

Ctrl + shift + F3 = Results, export to text from this window

Trigonometric derived nodal root angles correcting for stem width are more precise and easier to measure than traditional sweep angles

Larry M York, April 4, 2014

A subset of 12 images with varying stem widths were analyzed with several methods for measuring nodal root angle in order to demonstrate the influence of stem width on traditionally measured maize root crowns and to advance a quicker and more precise method for measuring a corrected nodal root angle. In past work, the stem is essentially held at the center of a protractor and the angle for a nodal root determined for only one side, or else averaged for both sides. Alternatively, a sweep angle (A_S) for the whole root system was measured in ImageJ using the angle tool, where the vertex of the rays is found at the center of the stem from where the focal nodal roots emerge (Figure 1). However, these methods fail to account for the influence of the stem width (W_S). One method to account for the stem width is to measure a left and right angle (A_{LR}) on either side of the stem. For both the left and right angle, a ray is formed from the end of a nodal root to its origin at the stem, and another from that origin and parallel to the growth direction of the stem (Figure 1) so the origin is the vertex of the angle. The summation of the left and right angle will be referred to as the corrected sweep angle for comparisons with the uncorrected sweep angle. When the stem width is zero the two sweep angles will be equal, however for a given stem width, the uncorrected sweep angle will be artificially large, or more shallow, relative to the corrected sweep angle.



The gray lines represent the stem and nodal roots of an idealized perfectly symmetric maize root crown. The dashed blue lines demonstrate the sweep angle (A_S), while the dashed purple lines demonstrate the left or right angles (A_{LR})

that are not influenced by the width of the stem (W_S). Measuring the width of the root system (W_{RS}) and the distance, or height (H), from W_S to W_{RS} allows trigonometric calculations of angles.

The corrected sweep angle is more precise than the uncorrected sweep angle, however, measuring the angle directly is difficult due to the need to imagine the growth trajectory of the stem (dashed, vertical purple line in Figure 1). A more straightforward method is to measure widths and heights (Figure 1) and to use trigonometry to calculate the angles.

The trigonometric sweep angle is calculated (equation 1) using the arctangent of the ratio of half of the width of the root system (W_{RS}) to the height (H). Notice this method bisects the sweep angle so the results must be multiplied by 2 to give the full sweep angle.

$$equation\ 1. \qquad A_S = 2 \arctan\left(\frac{W_{RS}}{2H}\right)$$

Similiarly, the trigonometric corrected sweep angle is calculated the same as the uncorrected sweep angle except the stem width (W_S) is subtracted from the width of the root system (equation 2). The corrected sweep angle is equivalent to the sum of the left and right angles (A_{LR}) and is not influenced by the width of the stem.

$$equation\ 2. \qquad 2\ A_{LR} = 2\ \arctan\left(\frac{W_{RS} - W_S}{2H}\right)$$

Alternatively, the angles may be expressed as degrees from horizontal with respect to the soil surface, in which case they are divided by 2 then subtracted from 90 degrees. However, this transformation only changes the presentation of the data and will not affect analysis.

Results

Influence of stem width on angle measurements

Uncorrected sweep angles were always more shallow than the corresponding corrected sweep angle (Figure 2). The difference between the uncorrected sweep angle and the corrected sweep angle increased with the stem width. Linear regression of the difference between sweep angle and corrected sweep angle against stem width revealed that for every 1 cm increase in stem width, there was a 4.77 degree increase in uncorrected sweep angle relative to the corrected sweep angle (R^2= .6907, p= 0.000809).

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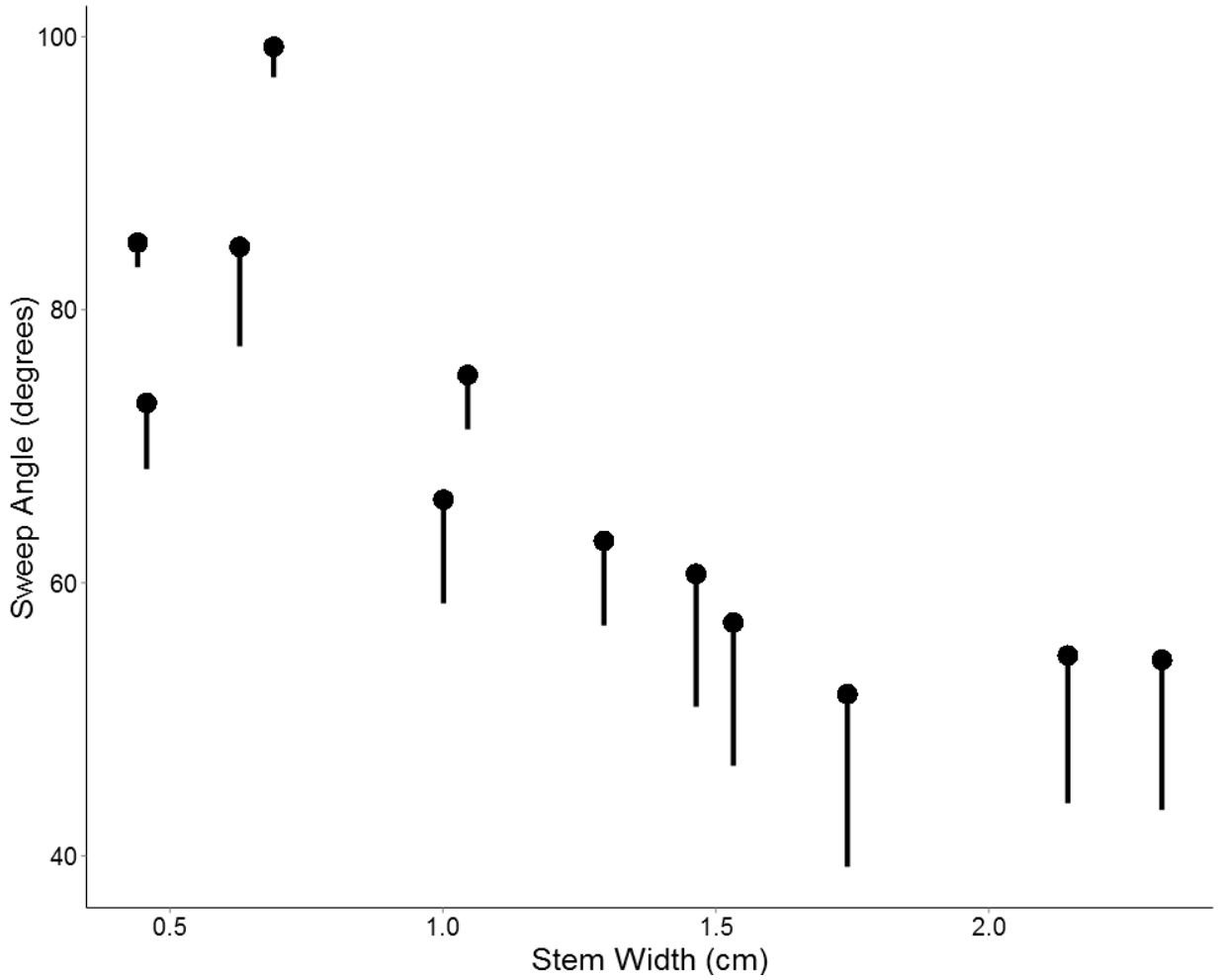
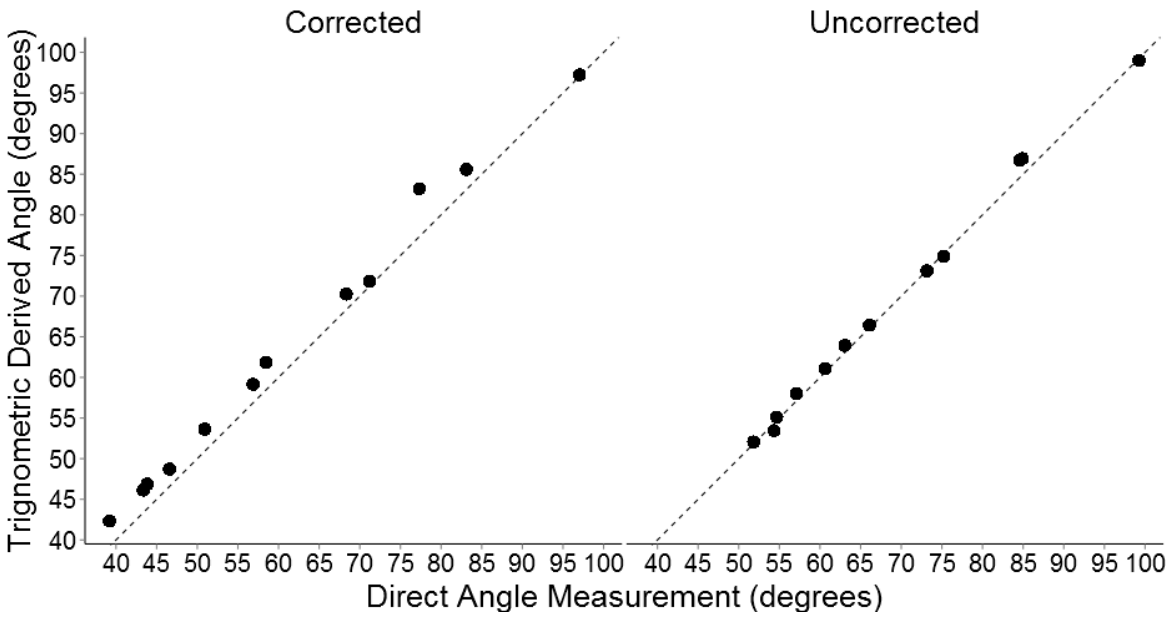


The sweep angle (point) and corrected sweep angle (bottom of line) are plotted against the stem width. The difference between sweep angle and corrected sweep angle is apparent because the length of the connecting line increases as the stem width increases.

Equality of direct angle measurements versus trigonometric calculated angles

The correlation between between direct sweep angles (DA) and trignometric calculated sweep angles (TA) was almost perfect (Figure 3, below). A 1:1 correlation between direct and trignometric correlations was observed for corrected sweep angle (TA = .97 DA + 3.69, r^2= .9948) and uncorrected sweep angle (TA = 1.01 DA - .49, r^2= .9983). The length measurements used for the trignometric sweep angle calculations are easier for the software user to measure than are direct angle measurements.



Both panels show the relationship between directly measured angles and angles derived using trigonometry. The panel on the left depicts the angle relationships for the corrected sweep angle, while the panel on the right depicts the relationships for the uncorrected sweep angle. In both panels, the dashed line represents a 1:1 relationship between directly measured and trigonometric derived angles. Note that the uncorrected angles are shifted about 10 degrees larger.