Comparative biomechanical characterization of maize brace roots within and between plants

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ABSTRACT:

As the world faces the challenge of feeding a growing population and increasingly unpredictable weather patterns, it is important to understand the plant features that will sustain food production under stress. One type of stress that plants experience is mechanical, and crops are susceptible to yield loss is by mechanical failure, called lodging. Brace roots (BR), aerial nodal roots of maize (Zea mays L.), are proposed to impart mechanical stability on plants, but little is known about the properties of BR that contribute to this function. Here, we define a 3-point bending method to test the comparative biomechanics of maize BRs within and between plants. We show that BR stiffness does not vary significantly within a plant neither along the length of a BR nor between BRs of different whorls. However, there are significant differences between plants of the same genotype. The differences manifested from variable BR diameter and thus nonideal span lengths for 3-point bending. The results presented here provide the foundation for widespread evaluation of BR mechanical properties and understanding their link to plant mechanical stability.

INTRODUCTION:

Importance of Maize to Agriculture

With the human population projected to reach 9.8 billion by 2050, farmers must maximize yields with increasingly limited resources. Compounded with this rising demand, climate change is jeopardizing yields by creating unpredictable weather patterns. Sudden and severe weather events can cause large patches of crops to either fall over or be blown over with wind, a phenomenon known as lodging. Lodging is responsible for between 10 and 66% of annual yield losses (Rajkumara 2008). There are two forms of lodging, root lodging and stalk lodging. Stalk lodging involves the breakage of the stalk at any point below the corn ear. Root lodging occurs when crops are up-rooted. Common causes of root lodging include strong winds, under-developed root systems, heavy rain, drought, and insect herbivory (Spike and Tollefson 1991; Elmore et al. 2005). The type of lodging and magnitude of risk is highly dependent on plant (Berry et al. 2004) and age (Carter and Hudelson 1988), with late season lodging resulting in significant yield loss. Despite the prevalence and impact of lodging, the plant phenotypes and physiological features that reduce the risk of late season crop lodging remain poorly understood.

Maize Brace Roots

Several cereal crops, including maize and sorghum, have structural organs called brace roots (BR). BRs are aerial nodal roots that have been theorized to brace the plants from mechanical loads and promote additional nutrient absorption (Wang et al. 1994; Van Deynze et al. 2018). A feature of BR cell walls is the deposition of lignin that is known to provide structure and stability (Hoppe et al. 1986). This lignification suggests that BRs are key structural components of plants and provide resistance to shear forces. However, it remains unknown how BRs contribute to plant stability and lodging resistance, or whether BR mechanical properties depend on genotype or environment. Here, we aimed to determine the within and between plant

variation of BR mechanical properties as the first step towards dissecting their role in structural stability.

Mechanical Characterization of Biological Materials

The American Society for Testing and Materials (ASTM) defines material testing standards for a variety of engineering materials including metals, polymers, ceramics, lumber, and composites, but not for living materials such as plants. The development of mechanical testing strategies requires an understanding of sample deformation under applied load such as tension, compression, shear, bending, and torsion. The mechanical characterization of plant tissues is particularly advantageous for investigating stress response and failure modalities involved in crop damage. Engineering theory and mechanical testing methodologies have been employed to examine the effects of mechanical stress on development in a variety of plants including tomatoes (Coutand et al. 2000), trees (Dean et al. 2002), barley (Kokubo et al. 1989) and maize (Robertson et al. 2015a, 2015b, 2016, 2017; Al-Zube et al. 2017, 2018; Stubbs et al. 2019). In particular, Robertson et al. (Robertson et al. 2015a) used mechanical testing in maize to investigate the interplay between stalk strength and stalk lodging resistance. Another study compared the mechanical properties of maize stems using different loading strategies: tension, compression, and 3-point bending (Al-Zube et al. 2018). Resulting elastic moduli were reproducible among the three testing approaches (test-to-test variation < 5%), but sample preparation involved in 3-point bending was the simplest. While these studies provide a foundation for the evaluation of BR mechanical properties, the small size of BRs compared to stalks introduces new challenges in mechanical testing.

This study aims to define a method to test the comparative biomechanics of maize BRs within and between plants. Using 3-point bending tests, we show that BR stiffness does not vary significantly within a plant, neither along the length of a BR nor between BRs of different whorls.

However, there are significant differences between plants of the same genotype. These results lay the foundation for understanding the link between BR biomechanics and plant stability.

MATERIALS AND METHODS:

Plant Material

Maize plants, genotype B73, were grown in a greenhouse to at least vegetative leaf stage 9 (V9). Plants were harvested by cutting the stalks with a pruning saw approximately 15 cm above the superior-most node with BRs and in the soil beneath the inferior-node with BRs. Plant cuttings were rinsed with water to remove soil and BRs were excised with a razor as close as possible to the stalk. BRs were dried (10-15% water weight) in a drying oven at 57 °C for 12 hours, to mimic conditions when maize is most susceptible to late season root lodging and to prevent water from introducing variability in results. For testing reproducibility along the BR length, three BRs per whorl per plant (18 BRs total) were chosen with the longest length and least curvature. Each BR was then cut into three smaller sections using a Dremel saw (3000 Series, 0.5 inch EZ lock stainless steel rotary blades). Sample dimensions (length, large diameter (*d1*) and small diameter (*d2*) were measured with a digital caliper (NEIKO 01407A, 0-6 inch, China). *d1* and *d2* measurements were taken at the midpoint of the BR section, because it is the loading site during 3-point bending. Selection of BR samples was limited by curvature, and selected samples ranged from 17.26 mm to 23.96 mm in length.

Naming Convention

A naming convention was defined to keep track of the BR sample classifications. Whorl (WR) was used to refer to a node with emerged BRs. Whorls were numbered from top to bottom of the plant with whorl 1 (WR1) as the superior-most WR with BRs present, and whorl 3 (WR3) the inferior-most whorl. Sections (S) 1, 2, 3 indicate the sample's location along the BR length

(with S1 being closest to the stalk, and S3 closest to the BR tip). Plants (P) were specified: P1, P2, and P3.

Mechanical Testing

A custom 3-point bend fixture was machined with 13.0 mm span length. Testing was conducted using an Instron 5695 (Norwood, Massachusetts USA) equipped with a 100 N load cell (Instron 2530 Series static load cell, Norwood, Massachusetts USA). Data were captured with Bluehill 3 software (Norwood, Massachusetts USA). Each BR sample was placed in the fixture and adjusted for mid-point loading. The specimen was tare loaded to approximately 0.2 N, gauge length was reset, and the test started. Specimen were loaded at a displacement rate of 1 mm/min. Testing was stopped upon fracture. Fracture was defined as the first observed crack in the BR section. This manifested in a sharp vertical drop in the force vs. displacement graph. Stiffness (k) was defined as the linear slope of the force vs. displacement curve and was extracted using a custom MATLAB code. To limit the subjectivity of the analysis, the linear slope was measured twice and averaged.

Analysis

Two-tailed unpaired t-tests were conducted to compare k values between sections, WRs, and plants. Sample size analyses were performed with G*Power, using the “Means: Difference from Constant (one sample case)” statistical t test, and “A priori: Compute required sample size – given alpha, power, and effect size”. Graphs were constructed in R statistical computing software with ggplot2 data visualization package (Wickham 2016). Regression analyses were conducted to decipher how independent variables (average diameter and span length) influenced variation.

RESULTS:

Stiffness Within Plants

To determine the variation in BR mechanical properties within a plant, we compared *k* for samples collected from 1) along the length of the BR and 2) between BRs of different whorls. The *k* of sections along the BR length, with S1 closest to the stalk and S3 closest to the BR tip, were not statistically different (**Fig.1A)** (2-tailed unpaired t test between: S1 & S2: P = 0.19, S2 & S3: P = 0.39, S1 & S3: P = 0.07). Further, the *k* of BR extracted from each of the whorls, with WR1 closest to the top and WR3 closest to the soil, were not statistically different between WR1, WR2, and WR3 **(Fig.1B)** (2-tailed unpaired t test between: WR1 & WR2: P = 0.79, WR2 & WR3: P = 0.66, WR1 & WR3: P = 0.88). This suggests that there is no statistical difference in BR biomechanics within plants.

Sample Geometry Variation

Although there was no significant difference in BR biomechanics within plants, there was a large distribution of *k*-values. To determine if this variation correlated with features of the samples themselves, *k* was regressed by average diameter (*d*) and ratio of ideal span length (**Fig.2**). As *d* increases, the *k* also increases in a direct linear relationship (R2 = 0.67) (**Fig.2A**).

We expect to see a distinct relationship between *k* and span length because diameter influences the ratio of span length to diameter. Due to the layering of the dermal tissues with the internal vasculature, we treated BRs as a “sandwich structured” polymer matrix composite (Al-Zube et al. 2018). According to ASTM Standards D726, ideal span length to sample diameter ratio for such composites is 16:1. This ratio was not attainable, because BR sample lengths were limited by natural curvature and variation of sample diameter. Instead, the span length of the mechanical testing fixture was machined to be 13 mm in length, because it was the longest span length possible for testing all BR samples. A constant span length resulted in the span length to diameter ratio for BR samples to range from 3:1 to 12:1.

To determine if the use of non-ideal span length ratios contributed to the variation in our results, we calculated the Actual Span Length to Ideal Span Length (*ASL:ISL*) ratio and regressed *k* by this ratio **(Fig.2B)**. *k*-values decrease with the increase of ASL:ISL, until ASL:ISL = 0.4, where the regression flattened out. At ASL:ISL = 0.4, in each 0.1 ASL:ISL increase, the sum of residuals decreases by a factor of 3. This indicated that as testing span length ≥ 40% of sample's ideal span length, sample *k*-value is less dependent on the testing span length. Thus both sample diameter and span length affect the measurement of *k.*

Stiffness Between Plants

To determine the variation of BR biomechanics between plants, we compared the distribution of test results from each plant. Color coding each data point by plant shows the measurements of diameters and *k* tend to cluster by plant (**Fig.2)**. Analysis of *k* between the three plants of the same genotype show significant differences between plants (**Fig.3**) (2-tailed unpaired t test between: P1 & P2: $P = 9.1 \times 10^{-6}$, P2 & P3: P = 0.81, P1 & P3: $P = 2.2 \times 10^{-8}$). This suggests that there is a statistical difference in BR biomechanics between plants.

DISCUSSION:

Stiffness Reproducibility Along Roots

It is difficult to test identical sections of BRs with repeatable results because of their short length and variably geometry. Curvature and warping of BRs is dependent on BR growth and the drying process, which may influence mechanical properties. Spatially restricting testing samples would result in many unusable sections. Although choosing the straightest sample in any section along the BR might be a feasible alternative approach, reproducibility among sections of the same BR was unclear. Here, we showed that comparative mechanical characterization of BRs is repeatable along the length of a BR.

Stiffness Reproducibility Between Whorls

We sought to reveal how *k* varied between BR WRs. Because BRs in WR3 were all touching the soil, and lateral roots were closer to the stem than in WR1 and WR2, it is difficult to obtain samples from these BR that are long and straight enough for testing. Here we determined that *k*-values were not significantly different between the three BR WRs. The longest, straightest sections of BRs can thus be chosen from any section on any WR in a plant of a particular genotype to obtain comparable *k*-values. This simplifies future studies involving the comparison of BR *k*-values between multiple genotypes.

Technical Variation: The Effect of Diameter and Span Length on BR Stiffness

Despite *k* between sections and WRs not varying significantly, there is variation within these data. Visualizing the relationship between *k* and BR average diameter (**Fig.2A**) shows as BR average diameter increased, so does the *k*. This relationship was directly linear; however, as $d > 2$ mm for each 0.5 mm increase of *d,* the sum of *k* residuals increased by a factor of 10. Higher diameters and consequently non-ideal span lengths enable shear forces that initiate slipping. Slipping introduces error that manifests in falsely high *k*-values.

Regression analyses describe the effect of BR geometry on *k* variability within samples. It revealed that ideal BR samples would have average diameters 2 mm or less and testing span lengths of at least 40% ideal span length. Due to biological variation and the wide range of BR sample geometry, limiting our analysis to just these ideal samples would restrict our ability to compare BR biomechanics.

Biological Variation: Stiffness Between Plants of the Same Genotype

Even though plants used in this study were grown from the same seed batch in identical controlled conditions, there is variation in BR geometry between plants. In order to compare results from different genotypes in futures studies, this biological variation can be overcome by

testing BR samples from a large enough population of plants. Indeed a sample size analysis suggests that 5 plants is sufficient to determine differences between B73 and another genotype.

Limitations

Here we utilize 3-point bending to determine *k*, because it is the easiest and quickest way to test large sample sizes, which are limited by length. Robertson et al. (Robertson et al. 2015b) highlighted one major pitfall of 3-point bending when loading maize stems that internode loaded stalks resulted in premature failure, a type of transverse deformation called brazier buckling. To acquire comparable *k* measurements and minimize brazier buckling, it is important to design bending experiments that cause material to fail on the surface experiencing tension - opposite anvil application. To confirm there was no brazier buckling in BR testing, macro images were taken every ten seconds of each 3-point bend test to qualitatively investigate any transverse deformation and other unusual loading patterns. Although brazier buckling was a concern, images showed that BRs failed opposite anvil application (data not shown). This most likely resulted because BRs, unlike maize rind, do not contain a spongy parenchymal pith.

The characterization of biological plant materials through theoretical engineering approaches is often difficult because the properties of plant materials depend on genetics and environment. Even when these independent variables are controlled for, there exists significant variation among properties and phenotypes. In order to develop standards for BR mechanical testing, we mitigated as many of these interfering variables as possible. Variation was minimized by using the same maize genotype (B73), growing all plants in the identical environments, harvesting at the same or similar growth stages, and drying all BRs in a drying oven for a controlled amount of time.

Despite these controls, several aspects of this methodology may have introduced slight variation in the results. First, samples were limited in length and number due to BR curvature. Several samples tested still exhibited slight curvature that may have contributed to inaccurate

increase in *k* before fracture. In addition to said possible sources of error, biological variability between maize plants was also unavoidable. For example, differences plant nutrient availability, energy diverted to fight pathogens, genetic variation, and growth timing can all manifest as differences in BR diameter and ideal span length, which may result in *k* variation.

Implications

At the intersection of plant biology and engineering, plant biomechanics is a quickly growing field. To facilitate its growth, methodologies for material testing and characterization should be clearly defined. We have shown that mechanical properties are reproducible and scalable along the BR. Overall, this research outlines a methodology that yields reproducible results, establishes a tolerance limit of BR diameter and span length ratios, and enables the comparison of BR biomechanics between different maize genotypes.

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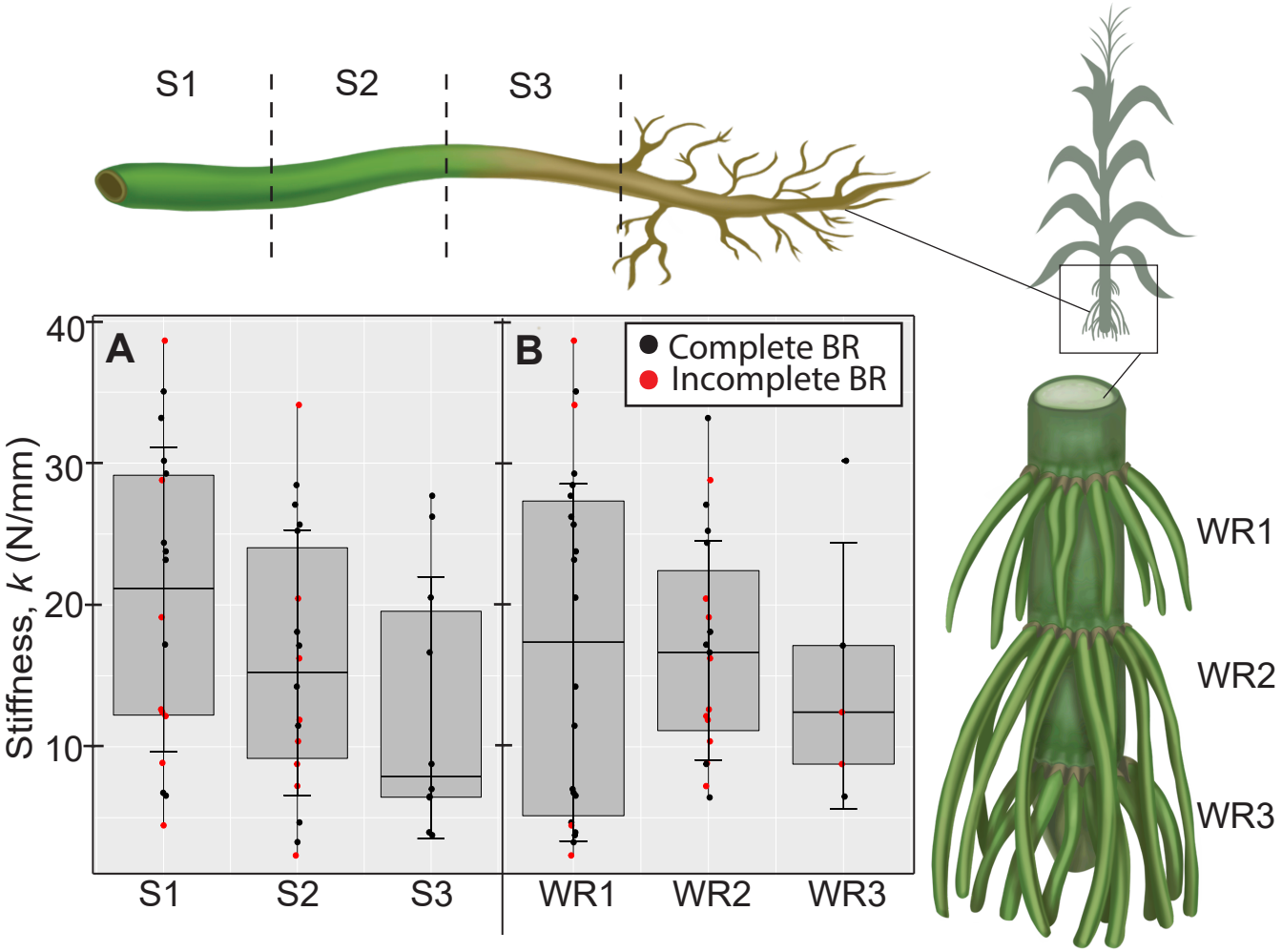

Fig.1 Stiffness *(k)* distribution plots reveal that there exists no significant variation between sections along a brace root nor between whorls of the same genotype. For 3-point bend tests, brace roots were cut into sections, with S1 closest to the stalk and S3 closest to the root tip. Brace roots were collected from three bottom whorls of each plant, with WR1 as the superior whorl of brace roots. *k* distribution plots were constructed for (**A**) three sections (S1, S2, S3) along the length of the brace root and (**B**) between whorls (WR1, WR2, WR3). There were no significant differences in *k* ($P > 0.05$) between sections along the root length (S1 & S2: $p = 0.19$, S2 & S3: $p = 0.39$, S1 & S3: $p = 0.07$), nor between whorls (WR1 & WR2: $p = 0.79$, WR2 & WR3: $p = 0.66$, WR1 & WR3: $p = 0.88$) [Unpaired t tests] Complete brace roots (BR) refer to those with S1, S2, and S3. Incomplete BR are those without S3.

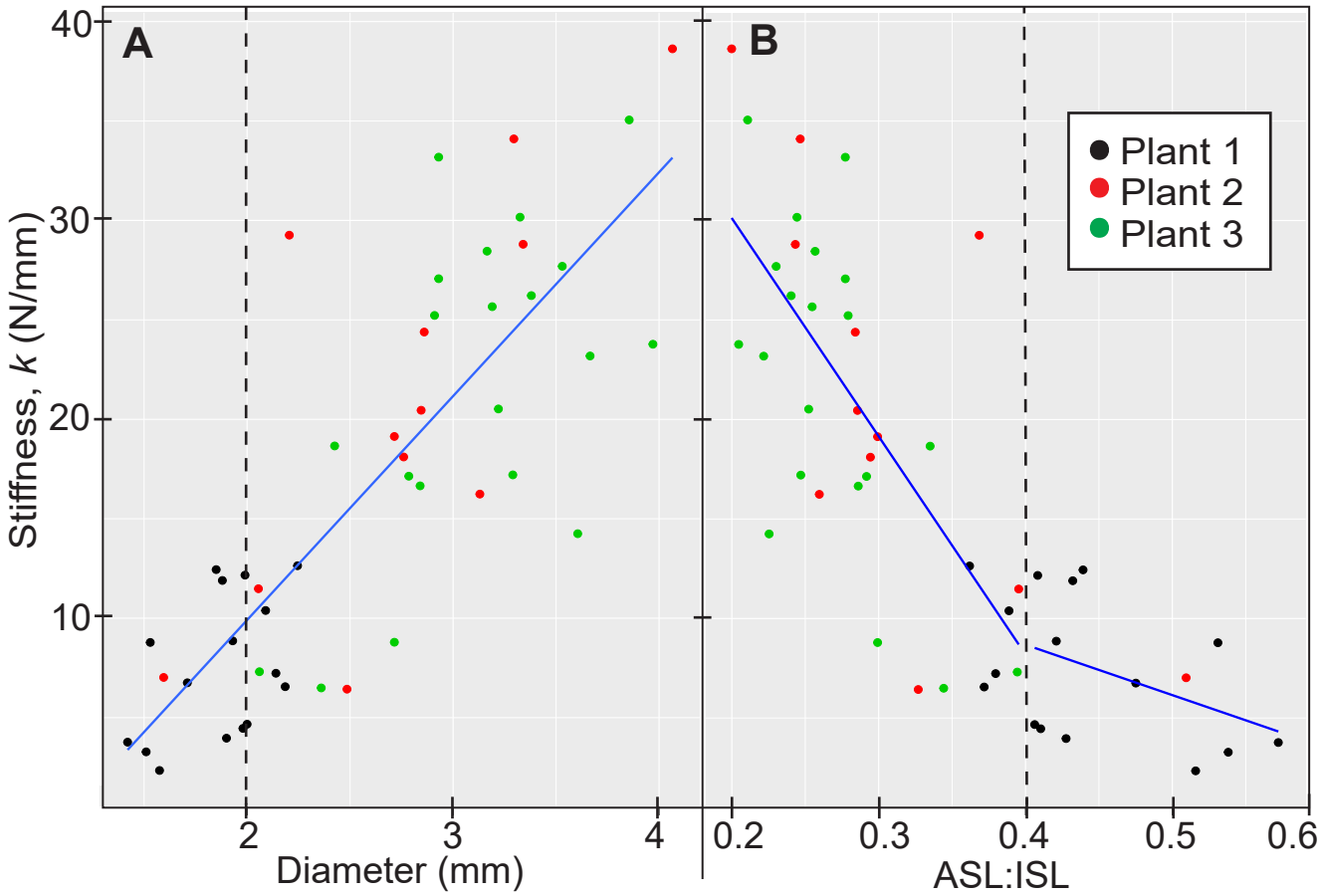

Fig.2 Variation in stiffness *(k)* is linearly correlated with changes in sample diameter and ratio of actual span length to ideal span length. *k* was regressed by (**A**) brace root average diameter (*d*) and (**B**) ratio of actual span length (13 mm) to ideal span length (16*d*) (ASL:ISL). The resulting regressions show that increases in diameter linearly correlate with increases in *k* (R^2 = 0.67). As average diameter reaches (*d* > 2 mm), sum of residuals for each *d* + 1 mm step increases by at least a factor of 10. Non-ideal spans (0 - 0.39% ideal) accumulate error and *k*-values are highly effected by nonideal span lengths until about 40% ideal. As ASL:ISL > 0.4, sum of residuals for each ASL:ISL + 0.1 decreases by at least a factor of 3 and the ASL:ISL is inversely correlated with *k*. This analysis shows that brace roots with *d* ≤ 2 mm and span length ≥ 40% (ASL:ISL ≥ 0.4) will yield the most reproducible stiffness (Dashed line).

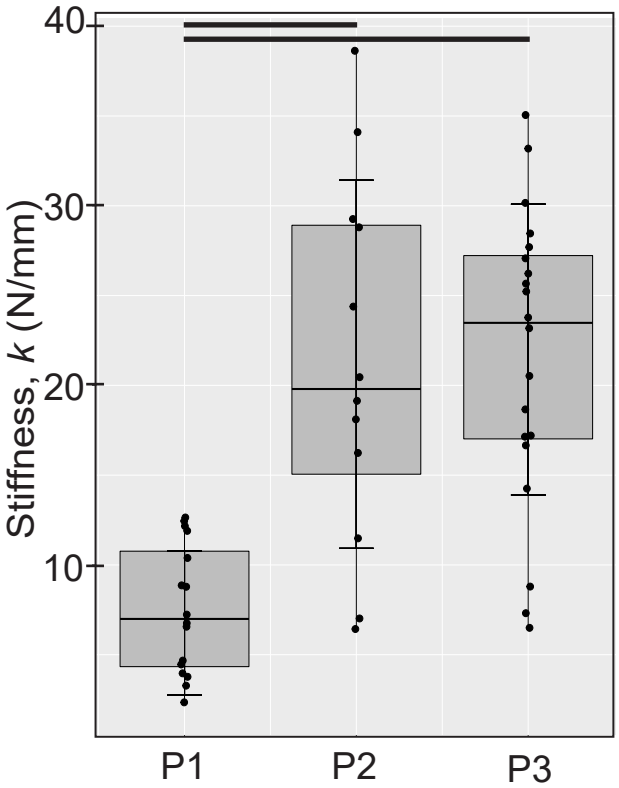

Fig.3 Stiffness *(k)* distribution plots between brace root samples grouped by plant show significant differences. P1 exhibited significantly lower *k*-values ($P < 0.05$) than P2 and P3 (P1 & P2: $p = 9.1 \times 10^{-6}$, P2 & P3: $p = 0.81$, P1 & P3: $p = 2.2 \times 10^{-8}$) [Unpaired t tests].