

Effect of Puncture Needle Diameter and Calibration Method on Blade Puncture Force and Mechanical Strength: Postprint

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Abstract

Leaf puncture force is an important indicator of leaf mechanical resistance, but the final results are influenced by needle diameter and calibration methods. To investigate the effects of different puncture needle diameters and calibration methods on leaf puncture force measurements, this study comparatively examined differences in leaf puncture force measured under different needle diameter and calibration conditions, as well as correlations with functional traits, in 30 monocotyledonous species with parallel venation and dicotyledonous species with reticulate venation on the campus of Guangxi University. Results showed: (1) When calibrated using puncture needle circumference, cross-sectional area, and leaf thickness, leaf puncture forces measured by different diameter needles varied under different calibration methods. (2) Under different calibration methods, the differences among puncture forces measured using three needle diameters varied. Specifically, when calibrated using only puncture needle circumference or using both needle circumference and leaf thickness, no significant differences in leaf puncture force were observed among needles of different thicknesses; when calibrated using only leaf thickness, thicker needles resulted in greater puncture force; when calibrated using only puncture needle area or using both needle area and leaf thickness, thicker needles resulted in smaller puncture force. The puncture forces of monocotyledonous plants measured with all three needle diameters were greater than those of dicotyledonous plants measured with needles of the same thickness. (3) The correlations between differences in puncture forces among the three needle diameters and leaf traits varied under different calibration methods; vein type, leaf density, leaf mass per area (LMA), cuticle thickness, and vein density may be structural factors influencing the differences in puncture forces among the three needle diameters. In summary, measurements of leaf puncture force using different diameter needles and calibration methods have different effects on plants with different vein types.

During leaf puncture force measurement, needle diameter and calibration methods need to be reasonably selected and standardized. When measuring leaf puncture force, among these three needles, a puncture needle with a diameter of 0.5 mm should be used for leaves with higher vein density and lower leaf mass per area, but is not suitable for harder leaves; a puncture needle with a diameter of 2 mm is not suitable for leaves with higher vein density and lower leaf mass per area; selecting a puncture needle with a diameter of 1 mm and calibrating using its circumference is relatively reasonable.

Full Text

Effects of Punch Needle Diameter and Calibration Methods on Leaf Force-to-Punch Mechanical Strength

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Abstract: Leaf force-to-punch is an important index reflecting leaf mechanical resistance, but the final results are affected by needle diameter and calibration method. To analyze the influence of different punch needle diameters and calibration methods on force-to-punch measurements, we measured differences in leaf force-to-punch among 30 species of parallel-veined monocots and reticulate-veined dicots from the Guangxi University campus under different needle diameters and calibration conditions, and examined correlations between these differences and functional traits. The results showed: (1) When calibrating using needle circumference, cross-sectional area, and leaf thickness, leaf force-to-punch varied with both needle diameter and calibration method. (2) The differences among force-to-punch values measured with three needle diameters varied across calibration methods. Specifically, when calibrated by needle circumference alone or combined with leaf thickness, no significant differences were observed among different needle diameters. When calibrated by leaf thickness alone, thicker needles yielded greater force-to-punch values. When calibrated by needle cross-sectional area alone or combined with leaf thickness, thicker needles yielded smaller force-to-punch values. Monocot force-to-punch values were greater than those of dicots at all three needle diameters. (3) Correlations between differences in force-to-punch across the three needle diameters and leaf traits varied with calibration method. Leaf venation type, leaf density, leaf mass per area, cuticle thickness, and vein density may be structural factors influencing these differences. In conclusion, needle diameter and calibration method significantly affect leaf force-to-punch measurements differently across plant groups with different venation types. We recommend careful selection and standardization of needle diameter and calibration methods. Specifically, among the three needles tested, the 0.5 mm needle is suitable for leaves with high vein density and low leaf mass per area but not for rigid leaves, while the 2 mm needle is unsuitable for leaves with high vein density or low leaf mass per

area. We recommend using the 1 mm needle calibrated by its circumference as the most reasonable approach.

Keywords: leaf force-to-punch, force-to-punch differences, leaf density, leaf mass per area, cuticle thickness, vein density

Introduction

How plants respond to environmental change has long been a central question in ecological research, and studies on plant functional traits and their relationship with the environment are considered an important approach for exploring frontiers in ecological science (Liu & Ma, 2015; Zhang et al., 2018). As the primary organ for photosynthesis and transpiration, leaf functional traits are a series of characteristics closely related to plant reproduction, survival, growth, mortality, and environmental adaptation (Liu & Ma, 2015; Wu et al., 2022). Leaves exposed to natural environments rely on mechanical resistance as a key trait to protect against abiotic damage from wind, rain, drought, and friction, as well as biotic damage from herbivore chewing and trampling (Niklas, 1992; Onoda et al., 2008; Enrico et al., 2016). Investigating the relationship between leaf functional traits and mechanical resistance helps us understand plant adaptation strategies to their habitats and represents a hot topic in plant ecology under global climate change. Numerous studies have examined the relationship between leaf structure and mechanical resistance, demonstrating that leaf density (Onoda et al., 2011; Méndez-Alonzo et al., 2019), leaf thickness and leaf mass per area (Onoda et al., 2011), cuticle thickness (Onoda et al., 2012), vein density (Roth-Nebelsick et al., 2001; Kawai et al., 2016), and venation patterns (Roth-Nebelsick et al., 2001; Enrico et al., 2016) are structural foundations affecting leaf mechanical resistance. These traits continue to receive sustained attention from researchers (He et al., 2019; Hua et al., 2020; Wei et al., 2022).

Previous studies commonly used punching, tearing, and shearing tests to measure leaf mechanical resistance (Read & Sanson, 2003; Onoda et al., 2011; Enrico et al., 2016), with many researchers employing these methods across different plant species. Leaf force-to-punch is an important indicator of resistance to mechanical damage, and calibration using needle diameter is a crucial step for standardizing measurements. Studies generally calibrate mechanical strength using needle circumference, cross-sectional area, and leaf thickness; however, needle diameters and calibration methods vary across studies. Onoda et al. (2008) measured force-to-punch in a *Plantago* species using a flat-ended cylindrical punch with a diameter of 1.345 mm, while Lusk et al. (2010) used a 2 mm punch for 13 tropical rainforest evergreen species, and Arellano-Rivas et al. (2018) measured force-to-punch in 23 species using a 1 mm cylinder. Other researchers (Read & Sanson, 2003; Wang et al., 2021; Meng et al., 2022) used 0.5 mm punches, and many (He et al., 2019; Hua et al., 2020; Wang et al., 2021; Wei et al., 2022) used 0.6 mm needles. Studies have calibrated force-to-punch using needle

circumference (Lusk et al., 2010; Wang et al., 2021; Wang et al., 2021; Wei et al., 2022) or cross-sectional area (Read & Sanson, 2003; Onoda et al., 2008). Additionally, He and Sun (2016) measured leaf force-to-punch in 107 species using a 1.2 mm cylindrical punch, Salgado-Luarte et al. (2022) used a 1.7 mm blunt rod, and Matsuda et al. (2022) measured leaf toughness with a 3 mm punch column, though these studies did not specify their calibration methods. Needle circumference and cross-sectional area characterize the contact surface where external force penetrates the leaf, and research has shown that punching strength is related to leaf thickness (Read & Sanson, 2003; Onoda et al., 2008). Currently, there is no standardized approach to needle diameter and calibration methods for leaf force-to-punch measurements, and no studies have compared multiple needle diameters.

Previous research has examined leaf mechanical strength differences between monocots and dicots. Dominy et al. (2008) found that monocot force-to-punch and shearing force were higher than those of dicots. Onoda et al. (2011) reported that parallel-veined monocots were more resistant to tearing than dicots, and Enrico et al. (2016) showed that reticulate-veined leaves had lower tearing force than parallel-veined leaves, with shearing force also differing significantly between the two venation types. Generally, monocots have parallel venation while dicots have reticulate venation. Combined with existing research, venation type affects leaf mechanical resistance, yet studies are lacking on how different needle diameters and calibration methods affect force-to-punch measurements in parallel-veined monocots versus reticulate-veined dicots.

This study was conducted on the Guangxi University campus, using 30 species of parallel-veined monocots and reticulate-veined dicots as research subjects. Using one-way ANOVA, independent samples t-tests, and correlation analysis, we measured leaf force-to-punch with three different needle diameters and calibrated the results using needle circumference, cross-sectional area, and leaf thickness. We also measured a series of leaf traits including leaf mass, leaf area, leaf mass per area, leaf thickness, cuticle thickness, leaf density, and vein density. Our objectives were to explore: (1) whether leaf force-to-punch differs when measured with different needle diameters and calibration methods; (2) whether leaf force-to-punch differs between parallel-veined monocots and reticulate-veined dicots; and (3) the relationship between force-to-punch differences and leaf traits, to provide reference for selecting appropriate needle diameter and calibration methods in future studies.

1. Materials and Methods

1.1 Study Site Description

The study was conducted on the campus of Guangxi University, located in Xixiangtang District, Nanning City, Guangxi (107°45′–108°51′ E, 22°13′–23°32′ N). Nanning has a subtropical monsoon humid climate with abundant sunshine

and rainfall. The average annual temperature is approximately 21.6 °C, with the hottest months being July and August (28.2 °C) and the coldest month being January (12.8 °C). Mean annual precipitation is 1,304.2 mm, concentrated between May and September, with an average annual relative humidity of 79%.

1.2 Measurement Methods

Between July and August 2021, we selected 15 parallel-veined monocot species and 15 reticulate-veined dicot species as research subjects (Table 1). For each species, 4–5 healthy mature leaves were collected from 6 different individuals.

1.2.1 Leaf Force-to-Punch Mechanical Resistance Measurement Leaf force-to-punch was measured using a small-scale tensile testing machine (ZQ 990, China). Leaf thickness was measured with a spiral micrometer, avoiding the main vein. Flat-ended cylindrical punch needles with diameters of 0.5 mm, 1 mm, and 2 mm were used to penetrate leaves while avoiding the midrib, with the maximum force reading recorded as leaf force-to-punch (Fp) (Onoda et al., 2011). Based on previous research on mechanical strength calibration and factors affecting mechanical strength, we applied five different calibration methods to the measured force-to-punch values, yielding five calibrated indices:

$$Fp1 = \frac{Fp}{C} \quad (\text{Lusk et al., 2010; Onoda et al., 2011})$$

$$Fp2 = \frac{Fp}{C \times LT} \quad (\text{Lusk et al., 2010})$$

$$Fp3 = \frac{Fp}{LT} \quad (\text{Choong et al., 1992; Dominy et al., 2008})$$

$$Fp4 = \frac{Fp}{A} \quad (\text{Read \& Sanson, 2003; Onoda et al., 2008})$$

$$Fp5 = \frac{Fp}{A \times LT} \quad (\text{Read \& Sanson, 2003})$$

where C represents needle circumference, LT represents leaf thickness, and A represents needle cross-sectional area.

1.2.2 Leaf Density and Leaf Mass Per Area Measurement After removing petioles, fresh leaf mass was measured with an electronic balance. Leaf area was determined using a leaf area meter (LI-3000C, LI-COR, USA). Leaves were then placed in labeled envelopes and oven-dried at 70 °C for 48 hours before dry mass was measured. Leaf mass per area (LMA) was calculated as leaf dry mass divided by leaf area, and leaf density was calculated as LMA divided by leaf thickness (Wei et al., 2022).

1.2.3 Leaf Anatomical Measurements Hand sections of leaves (avoiding the main vein) were made with double-sided blades to prepare temporary mounts. Upper and lower cuticle thicknesses were observed under an optical microscope (LEICA DM3000 LED, Germany) at 40 \times magnification, photographed, and measured using ImageJ 1.52n software (Rueden et al., 2016; www.imagej.nih.gov, USA).

Vein density measurement: A small leaf section (~1 cm²) was excised avoiding the main vein and placed in a 1:1 mixture of glacial acetic acid and 30% hydrogen peroxide. The sample was heated in a 70 °C water bath until nearly transparent, then rinsed with tap water, mounted on a slide, stained with safranin solution, gently rinsed with 50% ethanol, and photographed under an optical microscope. Vein length was measured using ImageJ software, and vein density was calculated as vein length per unit area (Xu et al., 2020).

1.2.4 Data Analysis Data were initially organized using Microsoft Excel 2010. Bar chart results are presented as means. One-way ANOVA was performed using IBM SPSS Statistics 23 (IBM, USA) to compare leaf force-to-punch among the three needle diameters, with Tukey's HSD test for multiple comparisons. Independent samples t-tests were used to compare leaf force-to-punch between monocots and dicots. Pearson correlation analysis was used to examine relationships between differences in force-to-punch across the three needle diameters and leaf traits. Two metrics were used to represent these differences: *Fp* (the range between maximum and minimum values across the three needle diameters) and *Fp SD* (the standard deviation of force-to-punch across the three diameters). SigmaPlot 14.0 (Systat Software, USA) was used for figure preparation.

2. Results

2.1 Effects of Needle Diameter and Calibration Method on Leaf Force-to-Punch

As shown in Figure 1 and Attached Figure 1, among the five calibration methods, Fp1 and Fp2 (Figure 1) exhibited significant differences ($P < 0.05$) between 0.5 mm and 2 mm needles in approximately two-thirds (67%) of species, while one-third (33%) showed no significant differences among the three needle diameters. In Fp3, Fp4, and Fp5 (Attached Figure 1), nearly all species showed significant differences among the three needle diameters. Overall, in Fp1, Fp2 (Figure 1: A, B; Figure 2: A, B) and Fp4, Fp5 (Attached Figure 1: B, C; Figure 2: D, E), the 0.5 mm needle produced relatively higher force-to-punch values, while the 2 mm needle produced relatively lower values. In contrast, for Fp3 calibrated by leaf thickness alone (Attached Figure 1: A; Figure 2: C), the 2 mm needle produced relatively higher force-to-punch values, while the 0.5 mm needle produced relatively lower values.

2.2 Differences in Leaf Force-to-Punch Between Monocots and Dicots Across Needle Diameters and Calibration Methods

One-way ANOVA and independent samples t-tests revealed that in Fp1 and Fp2 (Figure 2: A, B), no significant differences ($P > 0.05$) were found among the three needle diameters within either monocots or dicots. In Fp3 (Figure 2: C), both monocots and dicots showed significantly greater force-to-punch with the 2 mm needle compared to the 0.5 mm and 1 mm needles ($P < 0.05$). In Fp4 and Fp5 (Figure 2: D, E), the 0.5 mm needle produced significantly greater force-to-punch than the 1 mm and 2 mm needles ($P < 0.05$). In monocots, no significant differences were observed between the 1 mm and 2 mm needles, while in dicots under Fp5, all three needle diameters differed significantly, with the 0.5 mm needle producing the highest values and the 2 mm needle the lowest (Figure 2: E).

Monocot force-to-punch values were greater than those of dicots at all three needle diameters. The differences between monocots and dicots reached significant levels in Fp1 and Fp4 ($P < 0.05$) and highly significant levels in Fp2, Fp3, and Fp5 ($P < 0.01$) (Figure 2).

2.3 Relationships Between Differences in Force-to-Punch Across Needle Diameters and Leaf Traits Under Different Calibration Methods

Pearson correlation analysis revealed that in Fp1, no significant relationships ($P > 0.05$) were found between the range of force-to-punch values across the three needle diameters and any measured leaf traits in parallel-veined monocots. In reticulate-veined dicots, the force-to-punch range was positively correlated with leaf mass per area, upper cuticle thickness, and lower cuticle thickness (Figure 3; Attached Figure 2: B, C, D), suggesting that under Fp1 calibration, leaf mass per area and cuticle thickness may be factors causing these differences in dicots, while no leaf traits influenced this variation in monocots.

In Fp2, the force-to-punch range in parallel-veined monocots showed a significant positive correlation with leaf density (Figure 4; Attached Figure 3: A), while in reticulate-veined dicots, vein density was positively correlated with the force-to-punch range (Figure 4: E) but showed a weaker relationship with the standard deviation across needle diameters ($P > 0.05$) (Attached Figure 3: E). These results indicate that under Fp2 calibration, leaf density may be the structural factor causing differences in monocots, while vein density may be the factor for dicots.

In Fp3 (Figure 5; Attached Figure 4: A), the force-to-punch range in monocots was positively correlated with both leaf density and leaf mass per area, suggesting these traits may cause the observed differences under Fp3 calibration. In dicots, none of the six leaf traits showed significant correlations, indicating leaf traits did not affect these differences. In Fp4 (Figure 5; Attached Figure 4: B), the force-to-punch range in monocots was positively correlated with leaf mass per area, while in dicots it showed highly significant positive correlations

with leaf mass per area, upper cuticle thickness, and lower cuticle thickness. Across all 30 species, the range was significantly correlated with leaf mass per area and cuticle thickness, indicating these are likely structural factors causing differences under Fp4 calibration.

In Fp5, leaf density showed a positive correlation with the force-to-punch range among the six measured leaf traits (Figure 5; Attached Figure 4: C), suggesting leaf density is likely the structural factor causing differences under this calibration method.

3. Discussion

3.1 Impact of Needle Diameter and Calibration Method on Leaf Force-to-Punch

Our results demonstrate that both calibration method and needle diameter significantly affect leaf force-to-punch measurements. Overall, under Fp1, Fp2, Fp4, and Fp5 calibration methods, finer needles produced relatively higher force-to-punch values while thicker needles produced lower values. In contrast, under Fp3 (leaf thickness calibration alone), thicker needles produced relatively higher force-to-punch values while finer needles produced lower values. In parallel-veined monocots, palm species showed relatively high force-to-punch values, consistent with previous findings of strong palm leaf resistance (Dominy et al., 2008; Onoda et al., 2011). In reticulate-veined dicots, interspecific differences were generally smaller, though *Camellia*, *Osmanthus*, and *Ficus elastica* showed relatively high values while other species showed lower values.

Notably, when using the 2 mm needle to measure *Ophiopogon*, *Phoenix roebelenii*, *Alpinia zerumbet* ‘Variegata’, *Dianella tasmanica* ‘Variegata’, and *Ficus elastica*, force-to-punch readings exceeded 6 N, triggering force protection mechanisms and causing the needle to fail to retract automatically. This likely occurred because these coriaceous leaves have dense mesophyll tissue, creating greater friction against the needle. Additionally, the 2 mm needle failed to puncture the rigid leaves of *Dianella tasmanica* ‘Variegata’, merely creating an indentation, while the 0.5 mm needle bent easily during measurement, similar to findings that thinner biopsy needles are prone to deformation (Peng et al., 2021). Therefore, leaf rigidity should be considered when selecting needle diameter for force-to-punch measurements.

3.2 Differences in Leaf Force-to-Punch Between Monocots and Dicots Under Different Measurement and Calibration Methods

Our results indicate that needle diameter affects leaf force-to-punch differently in monocots versus dicots, with the magnitude of differences varying across calibration methods. Fp1 and Fp2 represent commonly used calibration methods for leaf mechanical strength. Under these methods, no significant differences

were found among the three needle diameters within either monocots or dicots, suggesting needle thickness does not affect leaf force-to-punch under Fp1 and Fp2 calibration. In contrast, under Fp3, Fp4, and Fp5, significant differences were observed between the 0.5 mm and 2 mm needles in both plant groups, indicating needle thickness affects leaf force-to-punch in both parallel-veined monocots and reticulate-veined dicots under these calibration methods.

Under Fp3 calibration by leaf thickness alone, the 2 mm needle produced significantly greater force-to-punch values than the 1 mm and 0.5 mm needles, likely because larger needle diameters have greater contact area with the leaf, resulting in increased friction and resistance.

Dominy et al. (2008) studied six monocot and ten dicot species in high-rainfall regions of Australia and found that monocot force-to-punch was higher than that of dicots. Onoda et al. (2011) similarly reported greater force-to-punch in monocots compared to woody dicots. Our study demonstrates that monocot force-to-punch values were significantly greater than those of dicots at all three needle diameters, confirming that monocots have higher leaf mechanical resistance than dicots and validating previous findings.

3.3 Relationship Between Differences in Force-to-Punch Across Measurement Methods and Leaf Traits

Our results show that correlations between the range of force-to-punch values across the three needle diameters and leaf traits differ between monocots and dicots under different calibration methods, indicating that structural factors causing these differences vary by plant group and calibration method. Overall, the differences were correlated with leaf density, leaf mass per area, cuticle thickness, and vein density, suggesting these are structural factors influencing variation across needle diameters. Many researchers have investigated relationships between leaf traits and mechanical properties, and our study further clarifies how measurement and calibration methods affect these relationships, contributing to a more comprehensive understanding of leaf ecological adaptation.

Previous studies have shown that leaf density is closely related to leaf mechanical properties (Méndez-Alonzo et al., 2013). Our results indicate that leaf density is a structural factor causing differences in force-to-punch across needle diameters under Fp2 and Fp3 calibration in monocots and under Fp5 calibration in both monocots and dicots. Kitajima and Poorter (2010) and Kitajima et al. (2012) studied tropical tree species in Bolivian forests and Panamanian wet tropical forests, respectively, concluding that leaf density enhances leaf toughness and extends leaf lifespan. Méndez-Alonzo et al. (2019) found that leaf density drives covariation between leaf hydraulics and biomechanics in 17 shrub species from California, demonstrating its influence on mechanical properties. These findings align with our results showing leaf density as a driver of differences across needle diameters, further validating its importance for leaf resistance.

Leaf veins, consisting of vascular tissue and surrounding mechanical tissue, pro-

vide transport, mechanical support, and defense functions (Wu et al., 2022). Kawai et al. (2016) studied relationships between vein traits and leaf mechanical properties in eight Fagaceae species from Japanese temperate forests, finding positive correlations between primary and secondary vein density and leaf mechanical resistance, indicating that vein density affects mechanical strength. Our study found vein density correlated with force-to-punch differences across needle diameters only in dicots under Fp2 calibration, suggesting it may be a structural factor influencing these differences. Additionally, venation type affects mechanical properties. Enrico et al. (2016) measured tearing and shearing forces in 72 Australian species, finding that parallel-veined leaves had higher tearing force than reticulate-veined leaves, with shearing force also differing between venation types. Our similar results demonstrate that leaf mechanical resistance is related to venation type, highlighting the importance of vein traits for leaf mechanics.

Leaf mass per area is an important functional indicator and is widely recognized as significantly correlated with leaf mechanical resistance (Kitajima & Poorter, 2010). The cuticle serves as a waterproof barrier protecting plants from desiccation and UV damage (Bourdenx et al., 2011) and is an important factor affecting leaf mechanical strength. Onoda et al. (2011) analyzed leaf mechanical properties and related traits across 2,819 species from 90 global locations, finding that thicker leaves with higher leaf mass per area had greater mechanical resistance. Onoda et al. (2012) also studied cuticles in 13 Australian evergreen woody species, finding that thicker cuticles increased mechanical strength and protected leaf surface integrity. Similarly, Wei et al. (2022) studied 14 dominant species in subtropical forests at different successional stages, reporting significant positive correlations between leaf mass per area and force-to-punch, and increased tearing force with greater cuticle thickness. These studies demonstrate the importance of leaf mass per area and cuticle thickness for leaf mechanics. Our findings show that leaf mass per area drives differences across needle diameters in monocots under Fp3 and Fp4 calibration, while leaf mass per area and cuticle thickness cause these differences in dicots under Fp1 and Fp4 calibration, further confirming their importance for mechanical strength. Notably, unlike Onoda et al.'s findings, we found leaf thickness may not be a driver of differences across needle diameters, possibly due to differences in growing environments, studied species, or sample sizes.

Previous research has shown that higher leaf density, greater leaf thickness, higher leaf mass per area, thicker cuticles, and greater vein density increase leaf mechanical resistance. Our results are generally consistent with these findings, though we found weaker correlations between vein density and force-to-punch differences across needle diameters ($r = 0.532$, $P < 0.05$) and no significant relationship with leaf thickness. Based on previous studies, we hypothesize that venation type, leaf density, leaf mass per area, cuticle thickness, and vein density are structural factors causing differences in force-to-punch across needle diameters. During force-to-punch measurements, these structural factors cause variation among needle diameters, with greater leaf density, leaf mass per area,

cuticle thickness, and vein density producing larger differences. Veins influence leaf mass per area, and both are important traits in the leaf economics spectrum, which predicts that higher vein density corresponds to lower leaf mass per area (Wu et al., 2022). Currently, increasing international interest exists in using large databases to test ecological hypotheses and improve vegetation models (Kattge et al., 2011; Enrico et al., 2016). As an important functional trait related to leaf construction cost (Wei et al., 2022), leaf mechanics research is crucial for understanding and enriching the global leaf economics spectrum. However, the lack of standardized needle diameters and calibration methods across studies may affect data integration efforts. Standardizing these methods would facilitate data compatibility and assist researchers in synthesizing datasets.

Our study examined the effects of three needle diameters and five calibration methods on leaf force-to-punch, revealing how different needle diameters and calibration methods affect measurements across plant groups with different venation types. We found that needle diameter affects measured force-to-punch values, that these effects differ across calibration methods, and that these differences correlate with leaf traits under various calibration methods, indicating that leaf traits are structural factors influencing variation across needle diameters. Parallel-veined monocots showed greater force-to-punch values than reticulate-veined dicots. For different plant types, we recommend selecting appropriate needle diameters: the 0.5 mm needle is suitable for leaves with high vein density and low leaf mass per area but not for rigid leaves, while the 2 mm needle is unsuitable for leaves with high vein density or low leaf mass per area. Based on previous research and our finding that circumference calibration produced no significant differences among the three needle diameters, we recommend using the 1 mm needle calibrated by its circumference for standardized measurements. Our results provide new insights into the relationship between needle diameter, calibration method, and force-to-punch, though further research across more families, genera, and habitats is needed to assess the generality of these findings and provide more comprehensive guidance for researchers selecting punch needles and calibration methods.

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Supplementary Figures

Attached Figure 1. Differences in leaf force-to-punch based on three needle diameters and different calibration methods in 30 species. Different letters indicate significant differences among needle diameters within each species ($P < 0.05$).

Attached Figure 2. Correlation between differences in leaf force-to-punch across three needle diameters and leaf traits for Fp1. *Fp1 SD* represents the standard deviation of force-to-punch across three needle diameters under Fp1 calibration. Black circles represent monocots, white circles represent dicots. M, D, and MD represent monocots, dicots, and all 30 species, respectively. $P < 0.05$ indicates significant correlation; $P < 0.01$ indicates highly significant correlation. The black dashed line shows the trend line for dicots.

Attached Figure 3. Correlation between differences in leaf force-to-punch across three needle diameters and leaf traits for Fp2. *Fp2 SD* represents the standard deviation of force-to-punch across three needle diameters under Fp2 calibration. The black solid line shows the trend line for monocots, and the red solid line shows the trend line for all 30 species.

Attached Figure 4. Correlations between differences in leaf force-to-punch across three needle diameters and leaf traits for Fp3, Fp4, and Fp5. *Fp SD* represents Pearson correlation coefficients between the range of force-to-punch values and leaf traits. * indicates significant correlation ($P < 0.05$); ** indicates highly significant correlation ($P < 0.01$); *** indicates extremely significant correlation ($P < 0.001$).

Note: Figure translations are in progress. See original paper for figures.

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