A new perspective on mechanical characterisation of Arabidopsis stems through vibration tests

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Abstract

The mechanical properties of plants are important for understanding plant biomechanics and for breeding new plants that can survive in challenging environments. Thus, accurate and reliable methods are required for the determination of mechanical properties such as stiffness and Young's modulus of elasticity. Much attention has been paid to the application of static methods to plants, while dynamic methods have received considerably less attention. In the present study, a dynamic forced vibration method for mechanical characterisation of Arabidopsis inflorescence stems was developed and validated against the conventional three-point bending test. Compared to dynamic tests based on free vibration, the current method allows to determine simultaneously more than one natural frequency, thus increasing the overall accuracy of the results. In addition, this method can be applied to the top parts of the stems that are more flexible, and where application of the three-point bending test is often limited. To demonstrate one of the potential applications of this method, it was applied to evaluate the influence of turgor pressure on the mechanical properties of Arabidopsis stems. Overall, the new dynamic testing approach has been shown to provide reliable data for the local mechanical properties along the Arabidopsis inflorescence stem.

Keywords: Arabidopsis, dynamic testing, mechanical properties, modulus of elasticity, multiple resonant frequency, vibration

1. Introduction

A key element of the Green Revolution in the 1950s and 1960s was the incorporation of dwarfing genes to breed plants with higher yields and shorter, stiffer straws that are less susceptible to lodging (Evenson and Gollin, 2003). An

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increase in global food production by 60% will be required by 2050 compared to 2005/2007 levels to feed the growing population of the world (Alexandratos and Bruinsma, 2012). To improve future food security mechanical as well as other physical/osmotic characteristics of plants have been identified as important (Connor, 2015). Understanding the mechanical properties of a plant, such as its modulus of elasticity, is therefore of high importance for breeding accessions that are more resilient to challenging environments.

In mechanical engineering there are a number of testing methods that can be used to determine the mechanical properties of materials and structures. In general, they can be divided into two groups: static and dynamic methods. In static tests, e.g., tensile and three-point bending tests, a uni-axial stress is applied to the studied specimen and its response, e.g., elongation or deflection, is recorded. The mechanical properties are then determined from analysis of the stress-stain relationship. In dynamic tests, e.g., impulse excitation of vibration (ASTM, 2015) and sonic resonance (ASTM, 2003), a stress is applied to excite a dynamic response of the specimen, based on which a mechanical characterisation is performed.

Static methods have been successfully adapted to study mechanical properties of various plants. The most widely used are the three-point bending (Ennos, 1993; Robertson et al., 2015; Al-Zube et al., 2018) and the four-point bending tests (Ennos et al., 2000; Robertson et al., 2015) that have been used on bamboo, banana petioles, giant reed, maize, sedge and other plant stems. The main advantage of these methods is the minimal preparation of the specimens required for testing.

Other types of static tests such as tensile (Greenberg et al., 1989; Al-Zube et al., 2018) and compressive tests (Al-Zube et al., 2017, 2018) have also successfully been applied to plants. However, they need a considerably higher amount of preparatory work compared to three- and four-point bending tests. A comprehensive overview of static mechanical tests for plants is given by Shah et al. (2017).

The main types of dynamic tests applied for the mechanical characterisation of plants are forced vibration and free vibration tests. Forced vibration tests have been applied to study the mechanical properties of plants since the pioneering works of Virgin (1955) and Burström et al. (1967). Niklas and Moon (1988) were first to use a multiple resonant frequency method to evaluate the flexural stiffness and the modulus of elasticity of a plant using a garlic flower stalk. This method was later applied to other plants and plant parts (Niklas, 1993, 1997). The free vibration method was utilised by Zebrowski (1991), Spatz and Speck (2002), Spatz and Theckes (2013) on winter wheat, triticale, giant reed and trees. In addition, vibration methods have found a number of applications on plants beyond the quantitative evaluation of their mechanical properties. For example, recently, free vibrations were utilised for the development of a non-destructive, high-throughput phenotyping method that can be applied on various plants (de Langre et al., 2019). An overview of vibrations in plants, including experimental methods for measuring them, is given by de Langre (2019).

Model plants are widely used in plant sciences to investigate and understand various processes and mechanisms in plants. *Arabidopsis thaliana* is widely used in this context due to its small size, short life cycle, and the availability of various mutants with altered parameters. In most cases, the mechanical characterisation of Arabidopsis stems is performed using static methods such as three-point bending (Paul-Victor and Rowe, 2010), four-point bending (Goubet et al., 2009), tensile (Ryden et al., 2003), and compression tests (Verhertbruggen et al., 2013). The testing methods used for mechanical characterisation of Arabidopsis are reviewed in Brulé et al. (2016).

Historically, dynamic methods have found limited application for mechanical characterisation of Arabidopsis stems, e.g., a forced vibration method was utilised for modal analysis for phenotyping (Der Loughian et al., 2014) and the measurement of bending stiffness was performed using free vibrations method (Nakata et al., 2018). The former method does not give the possibility to evaluate the modulus of elasticity, a key parameter for determining the overall mechanical properties of a plant stem. On the other hand, it may be possible to extend the method described by Nakata et al. (2018) to the determination of the modulus of elasticity by adding the evaluation of the mass and geometrical properties of the studied stem, but these steps are not discussed in the aforementioned work.

The aim of this paper is to present a new type of multiple resonant frequency dynamic testing method for mechanical characterisation of Arabidopsis inflorescence stems. The developed dynamic testing approach was validated against static three-point bending tests. The new dynamic method requires the same amount of preparation time on specimens as a three-point bending test and only one additional measurement, the mass of the stem, for processing of the results. However, compared to the static and free vibration-based dynamic methods that have previously been applied to plants, the presented method allows for multiple estimations of the modulus of elasticity to be determined, hence increasing the accuracy of the results. In addition, a lower level of deformation of the tested specimen is achieved compared to previous dynamic tests, through implementation of the clamped-clamped boundary condition. For the first time, tests were performed on different sections of the same stem, that, as expected, showed a clear difference between their mechanical properties. Moreover, the presented dynamic method provides reliable data for the upper part of the stem, that is more flexible and where static methods usually fail to provide the average mechanical properties. Understanding of these local differences in the mechanical properties along the stems is important for studying changes in plants due to various factors, e.g. thigmomorphogenesis or turgor pressure variation. In addition, insight into the variation of the mechanical properties will also help to inform the breeding of plants to be grown in extreme environments (e.g. subjected to high wind), where mechanical properties averaged over the whole stem are not sufficient to determine the susceptibility of the plants to lodging and other forms of mechanical damage.

2. Materials and methods

2.1. Plants

Arabidopsis thaliana seeds of ecotype Columbia-0 were sown in a single pot, which was kept for 48 hours at 4°C, prior to moving it to a growth chamber. The conditions in the growth chamber were as follows: long day cycle (16 h of light and 8 h of darkness), temperature at 22 °C, light intensity at 150 μmol m^{-2} s^{-1}, and humidity at 60 %. After 14 days, the seedlings were transplanted into individual pots (pot diameter = 76 mm) and were kept in the growth chamber for the next four weeks before the mechanical tests. At the time of the mechanical tests the plants were in the developmental stage where stems are mature and growth rate is reduced (Boyes et al., 2001); none of the plants showed any signs of senility. A total of 71 plants were used for the comparison of the dynamic and static testing methods. Taking into account that the mechanical properties of freshly cut Arabidopsis stem segments vary in time (Paul-Victor and Rowe, 2010) it was decided not to conduct both tests on the same segment. The plants were randomly separated into two groups and 40 plants were characterised using the dynamic method and 31 using the three-point bending test. For the study of the influence of turgor pressure on the mechanical properties of Arabidopsis inflorescence stems a separate group of 20 plants was grown under the same conditions.

2.2. Sample preparation

The tests were conducted on the primary inflorescence stem from which two segments were cut using a razor blade. The first segment was taken from the base of each stem and hereafter is referred to as "bottom part of the stem" while the second was taken from the tip of the stem and hereafter referred to as "top part of the stem". Both segments, where necessary, were cleared from branches, fruits, flowers, and young floral buds prior to the tests using the same razor blade and taking care not to damage the tested part. In addition, a 15-20 mm segment that contains the growth zone was removed from the apex of the top part of the stem. Paul-Victor and Rowe (2010) reported that 15 minutes after cutting, the loss in stiffness of Arabidopsis stems is around 10% due to loss in turgor pressure as a result of moisture evaporation. Taking into account that the time required for both tests in this study is significantly lower (less than 2 minutes from stem cutting to the end of the test), the stem ends were not sealed and all tests were performed immediately after the stem segments were cut.

In this study, the widely used approximation that an Arabidopsis stem segment has a circular cross-section of constant diameter along its length (see e.g., Turner and Somerville (1997), Bichet et al. (2001)) was adopted. Consequently, the second moment of area, I, of the stem segment is given by:

$$I = \frac{\pi}{64} D^4, \tag{1}$$

where D is the diameter of the stem cross-section. After each test, a photograph of the tested stem segment on a calibration ruler was taken using a USB digital microscope (UM012C, Mustech Electronics Co., Ltd). The diameter was determined using the ImageJ software (Schneider et al., 2012) as the averaged diameter over several locations along the tested segment.

2.3. Three-point bending tests

Three-point bending tests were performed using a Zwick Roell Z2.0 uni-axial tension compression machine (Zwick Testing Machines Ltd.) equipped with a 5 N load cell (Figure 1a). The anvil had a rounded end to minimise artificial cross-section deformation (Robertson et al., 2015) and was displaced at a speed of 2 mm min^{-1}. The distance between supports, L, was 50 mm. This value gives a span-to-depth ratio of the tested specimens, depending on the stem part, between 30 and 65. These values minimise the influence of shear on the measured deflection and are in line with recommendations by Shah et al. (2017). For calculation of the Young's modulus of elasticity, E, which characterises the ability of a material to resist elastic deformations, the slope, c, of the steepest linear part of the force-displacement curve was determined. This ensures that the response of the sample remains in its elastic regime. The bending rigidity, EI, which quantifies the ability of a material to resist bending, of the stem parts was determined as:

$$EI = \frac{L^3 c}{48}, \tag{2}$$

and the value of elastic modulus was then calculated as $E = EI/I$.

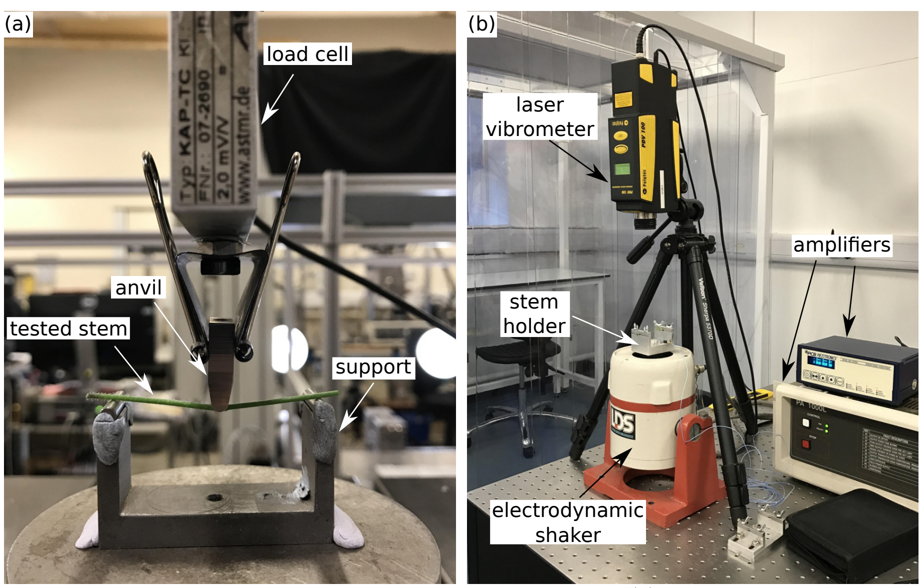


Figure 1: Experimental setups used for the mechanical tests. (a) Three-point bending test; (b) Vibration test.

2.4. Vibration tests

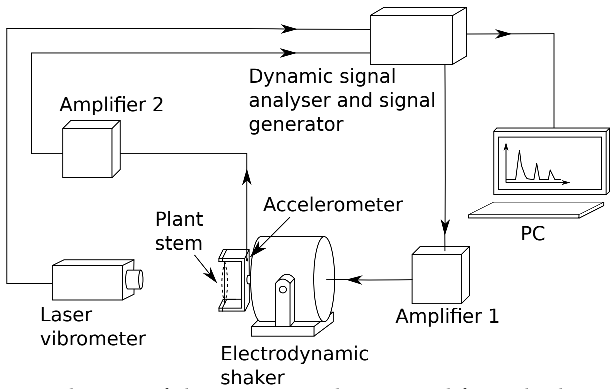


Figure 2: Schematic diagram of the experimental setup used for multiple resonant frequency dynamic tests.

Multiple resonant frequency dynamic tests, namely base excited vibration tests, in a clamped-clamped configuration were used to determine the mechanical properties of the stems from their natural frequencies. The experimental setup shown in figures 1b and 2 includes a dynamic signal analyser (Quattro, Data Physics, USA), a permanent magnet electrodynamic shaker (LDS V406) with an amplifier (LDS PA1000L), a piezoelectric accelerometer with amplifier (482C Series, PCB Piezoelectronics, USA) and a laser vibrometer (PDV 100, Polytec, Germany).

A random signal from the signal analyser is fed through the amplifier 1 (Figure 2) to the shaker. The accelerometer measures the acceleration of the base of the shaker and sends signal to the signal analyser through the amplifier 2. The laser vibrometer records the response of the tested structure to the applied vibrations and this signal is also fed to the signal analyser. The transfer function from the accelerometer and laser vibrometer signals is built using a specialised software for the signal analyser (SignalCalc, Data Physics, USA). A representative example of a transfer function is presented in figure 3. The peaks on the transfer function correspond to the natural frequencies (f_i) of the tested stems. The Young's modulus is then calculated from each value of f_i using the formula for the natural frequency of a beam, based on the Euler-Bernoulli beam theory (Blevins, 1979):

$$f_i = \frac{\lambda_i^2}{2\pi L^2}\sqrt{\frac{EI}{m}}, \quad i = 1, 2, 3, ..., n \tag{3}$$

where L - length of the stem, I - second moment of area, m - mass per unit length, and λ_i is a dimensionless parameter that is obtained from the characteristic equation corresponding to the applied boundary conditions and vibration mode. In the current setup, i.e. a clamped-clamped beam, tabulated values from Blevins (1979) have been used (see also table 1).

Table 1: Values of λ_i for different vibration modes from Blevins (1979)

i	1	2	3	4
λ_i	4.73004074	7.85320462	10.9956079	14.1371655

To evaluate the mass per unit length, m, the mass of the stem segment, cut from the holder using a razor blade, was determined using a precision balance and divided by the stem length (50 mm in this case) directly after the vibration test. This minimised changes in stem mass due to moisture evaporation.

The number of resonant frequencies that can be determined from this type of vibration test depends on the stiffness and size of the tested specimen and the resolution of the measurement system. The stiffer and shorter the tested stem, the higher are the values of its natural frequencies. Since the length of the tested stem segments is fixed, the number of determined natural frequencies depends on the stem diameter. From the vibration tests on the bottom part of the stem three natural frequencies were determined, while from tests on the top part of the stem four could be measured due to the lower rigidity of the top part of the stem.

The final value of the modulus of elasticity for each tested stem segment was determined as an average of the values obtained from each natural frequency.

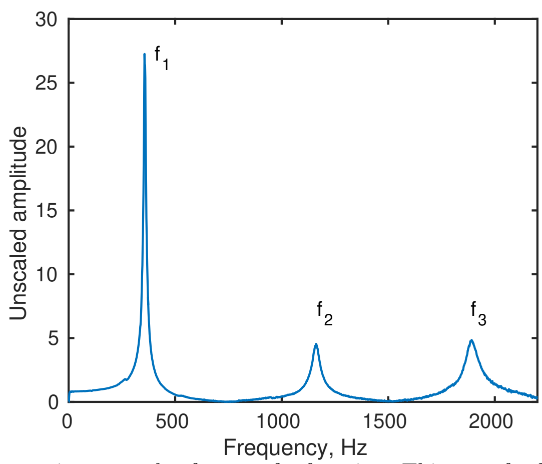


Figure 3: Representative example of a transfer function. This transfer function was obtained from one of the vibration tests performed on the bottom part of the stem. Peaks on the transfer function correspond to the natural frequencies of a tested stem.

2.5. Examining the influence of stem clamping in the vibration test holder

For a vibration test with clamped-clamped boundary condition it is necessary to fix both ends of the tested specimen in the holder to prevent both linear displacement and rotation. The simplest way to fix a stem in the holder is to squeeze both ends between clamps. However, this will damage the tissues of the stem ends due to flattening and could affect the measured values for the stem natural frequencies. Therefore, preliminary tests were conducted, with and without squeezing of the stem ends, on Arabidopsis inflorescence stems.

In order to prevent squeezing of the stem ends, spacers of different thickness, based on the diameter of the stem, were placed between the top and bottom clamps of the holder (figures 8a and b). This ensured secure fixation of the stem for testing without substantial damage to the stem ends from the clamping system. Since the damage by clamping is irreversible, first the stem was tested with the inserted spacers and straight after the test was repeated for the same stem but with spacers removed.

2.6. Investigation of influence of turgor pressure on the mechanical properties

To demonstrate a potential application of the presented dynamic method, it was applied to investigate the influence of turgor pressure on the mechanical properties of Arabidopsis stems. For this study 20 segments were cut from the bottom part of the Arabidopsis inflorescence stems as described in the sample preparation section. Immediately after cutting, segments were treated with carborundum powder (fine, about 180 grit) to create micro scratches on their surfaces making them permeable to the hyperosmotic solution that would be applied in the second stage of this test (see e.g. Cosgrove and Steudle (1981)). After the treatment, segments were submerged into distilled water prior to testing to prevent their dehydration. The baseline dynamic test was conducted according to the procedure described earlier. After the test, each stem was carefully removed from the holder's clamps and its mass and diameter were measured. The stems were then subjected to hyperosmotic stress (300 mM mannitol treatment) to decrease their cell turgor pressure. After one hour, the vibration tests were repeated to measure the natural frequencies of the stems with reduced turgor pressure. At this stage the evaluation of the mass of the stem segment was carried out again to account for a potential change in mass between the two tests. Finally, after the completion of the second test, the mass of the 50 mm segment between the clamps was measured.

2.7. Statistical analysis

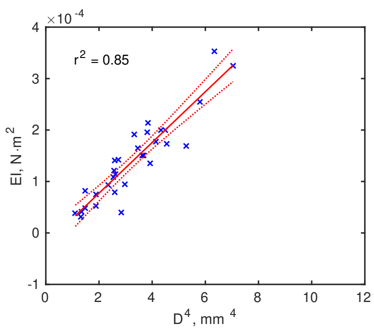
Differences in the modulus of elasticity values determined using static and dynamic methods for the same parts of the stem as well as differences between mechanical properties of bottom and top parts of the stems were investigated using a non-parametric Wilcoxon rank-sum test. The choice of this test is explained by small sample sizes of different lengths and by the fact that some of the data was not normally distributed. To examine the influence of stem clamping on the detected natural frequencies of the same stem tested with and without inserted spacers paired t-test was utilised. This test was also applied to study differences in the mechanical properties of the stems associated with the decrease of turgor pressure. Tests were carried out using Matlab (R2015b, MathWorks, USA) ranksum and ttest functions correspondingly. Statistically significant difference was established at $p \leq 0.05$.

3. Results

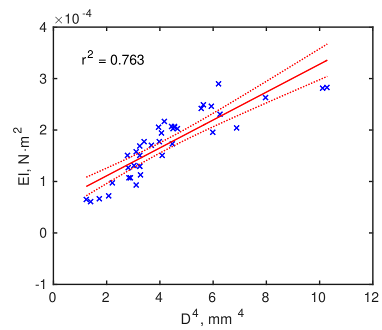
3.1. Bottom part of the stem

The bottom part of an Arabidopsis stem consists of mature tissues and cells that have stopped growing. Consequently, a more uniform distribution of the mechanical properties is expected along the length of the tested segment and variation between different stems is expected to be relatively low. Three natural frequencies were obtained from the vibration tests for this part of the stem based on which mechanical properties were determined.

In figure 4 bending rigidity (EI) of each tested stem is plotted against stem diameter raised to the fourth power (D^4) using data from both vibration and three-point bending tests. The EI values for the vibration test correspond to the average of the EI_i values computed from the determined natural frequencies f_i for each stem. In both cases, EI shows statistically significant positive correlation with D^4 ($r^2 = 0.85$ for three-point bending test and $r^2 = 0.763$ for vibration). In addition, standard deviations of the E values determined from each natural frequency in the vibration tests are small (figure 5a). These observations support the assumption that there is a low variation in the modulus of elasticity in the bottom part of the different tested stems.



(a)



(b)

Figure 4: Scatter diagrams showing bending rigidity vs stem diameter raised to the fourth power for the bottom parts of the Arabidopsis stems. (a) Scatter diagram based on the results of the three-point bending tests; (b) Scatter diagram based on the results of the vibration tests.

The mean values of the modulus of elasticity, together with standard deviations are presented in table 2. $\overline{E}_i$ represents the mean of all values of E determined from the i^{th} natural frequency, while $\overline{E}$ is the mean value of the modulus of elasticity averaged first over all values of E_i for each stem and then over all tested stems. The variation between $\overline{E_i}$ values is low. The small differences in the obtained values of $\overline{E}_i$ can be attributed to the fact that different mode shapes have participation from different sections of the stem segment. This together with any heterogeneity along the segment results in an uneven stress distribution.

Figure 5b shows that there is no statistically significant difference ($p > 0.8$) in the modulus of elasticity values obtained by the three-point bending and

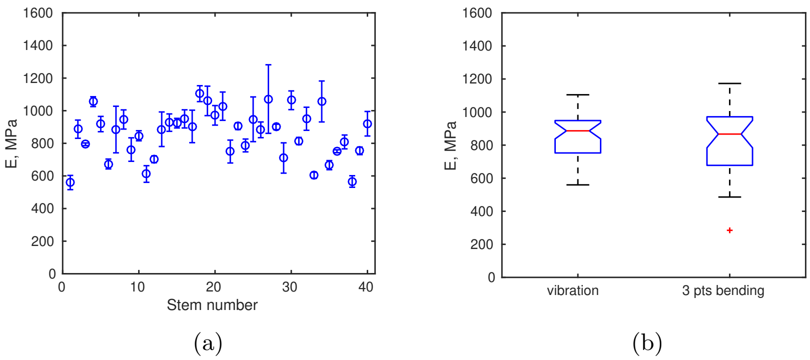


Figure 5: Modulus of elasticity determined for the bottom parts of the Arabidopsis stems. (a) Mean values of the modulus of elasticity, E, determined using the vibration method, errorbars represent standard deviations; (b) Box plots presenting E, determined from vibration (n=40) and three-point bending tests (n=31).

vibration methods for the bottom part of the stem. Both tests show good agreement in terms of mean values (difference $< 2\%$) and standard deviations (table 2). In case only the first natural frequency was used for the calculation of the modulus of elasticity, the discrepancy between the two methods would be higher (3-4%).

Table 2: Modulus of elasticity determined from mechanical tests for the bottom part of Arabidopsis inflorescence stems. Data is presented as mean $\pm$ standard deviation. $\overline{E}_1$, $\overline{E}_2$ and $\overline{E}_3$ mean values of the modulus of elasticity determined from corresponding natural frequencies in the vibration tests. $\overline{E}$ mean values of the elastic modulus.

type of the test	$\overline{E_1}$, MPa	$\overline{E_2}$, MPa	$\overline{E_3}$, MPa	$\overline{E}$, MPa
vibration	875 ± 156	872 ± 173	826 ± 150	858 ± 147
bending	-	-	-	842 ± 211

3.2. Top part of the stem

In contrast to the bottom part, the top part of Arabidopsis stems consists of young tissues and cells. In addition, along the length of the tested segments, variation of mechanical properties is expected, since closer to the tip tissues are younger compared to those at the lower end of the tested segment. The smaller diameter and lower rigidity of the top part of the stems allowed to determine the first four natural frequencies for each tested stem. These frequencies were used for calculation of the mechanical properties. However, it was not possible to test the top parts of some stems using the three-point bending technique because of their shape and excessive flexibility. This led to sagging of the stem segments under their own weight and slipping of the stem ends between supports during the tests. In addition, in some cases, the point of maximum deflection was not the same as the point of force application. In the present study, the three-point

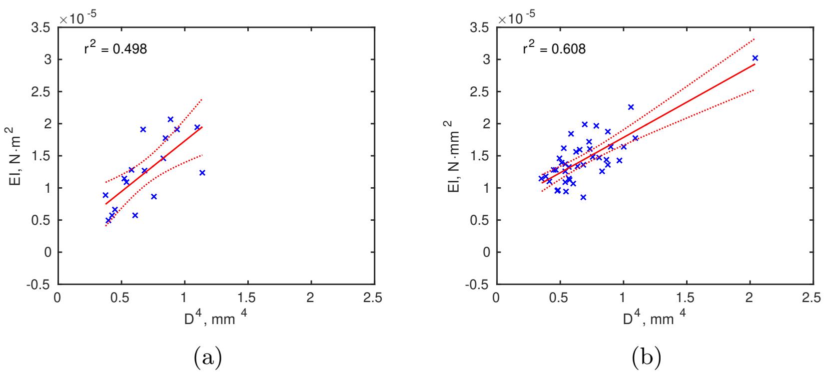


Figure 6: Scatter diagrams showing bending rigidity vs stem diameter raised to the fourth power for the top parts of the Arabidopsis stems. (a) Scatter diagram based on the results of the three-point bending tests; (b) Scatter diagram based on the results of the vibration tests.

bending test failed in approximately 45% of cases when applied to the top part of the stem. Thus, the results for this type of tests are given only for those specimens that could be tested. On the other hand it was possible to test all top parts of the stems using the vibration method due to the clamped-clamped boundary condition and the high resolution of the laser vibrometer.

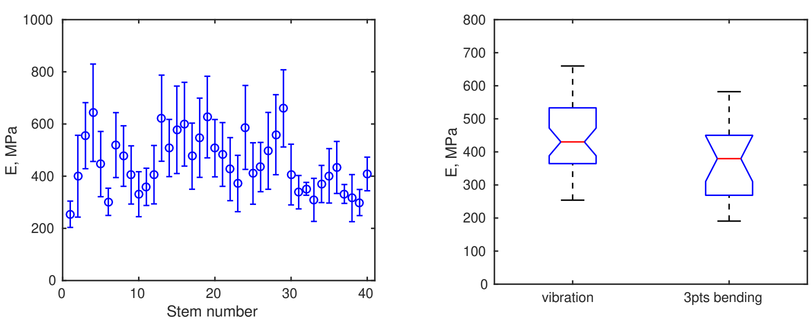
The bending rigidity for each tested top part of the stem is plotted against stem diameter raised to the fourth power in figure 6. For both tests there is weaker positive correlation ($r^2 = 0.608$ for the stems tested using vibration method, $r^2 = 0.498$ for the stems tested using three-point bending method) between these values which can be explained by the non-uniform properties of the tested segments compared to the bottom part of the stem. The standard deviations of the E values, determined from each natural frequency in the vibration tests, are significantly higher than those determined for the bottom part (figure 7a). This confirms that material properties along the top parts of the Arabidopsis stems are not uniform.

Values of $\overline{E_i}$ determined from each natural frequency (table 3) show a stronger variation compared to the bottom part of the stem associated with the non-uniform mechanical properties along their lengths. For the top part of the stem both tests show a statistically significant difference in the results ($p < 0.05$) (figure 7b). However, this can be attributed to the fact that even though three-point bending test provided results for 55% of the tested stems, this results might be biased by the same reasons that led to the failure of obtaining results in other 45% of tested stems.

Overall, values of the modulus of elasticity of the top part of the stem are significantly different ($p < 0.0001$) from those for the bottom part of the stem, showing approximately twice lower values. This, as expected, shows that the material properties of an Arabidopsis inflorescence stem vary along its length.

Table 3: Modulus of elasticity determined from mechanical tests for the top part of Arabidopsis inflorescence stems. Data is presented as mean ± standard deviation. $\overline{E_1}$, $\overline{E_2}$, $\overline{E_3}$ and $\overline{E_4}$ mean values of the modulus of elasticity determined from corresponding natural frequencies in the vibration tests. $\overline{E}$ mean values of the elastic modulus.

type of the test	$\overline{E_1}$, MPa	$\overline{E_2}$, MPa	$\overline{E_3}$, MPa	$\overline{E_4}$, MPa	$\overline{E}$, MPa
vibration	525 ± 161	384 ± 103	404 ± 146	482 ± 135	449 ± 108
bending	-	-	-	-	369 ± 109



(a) (b)

Figure 7: Modulus of elasticity determined for the top parts of the Arabidopsis stems. (a) Mean values of the modulus of elasticity, E, tested using the vibration method, errorbars represent standard deviations; (b) Box plots presenting E, determined from vibration (n=40) and three-point bending tests (n=17).

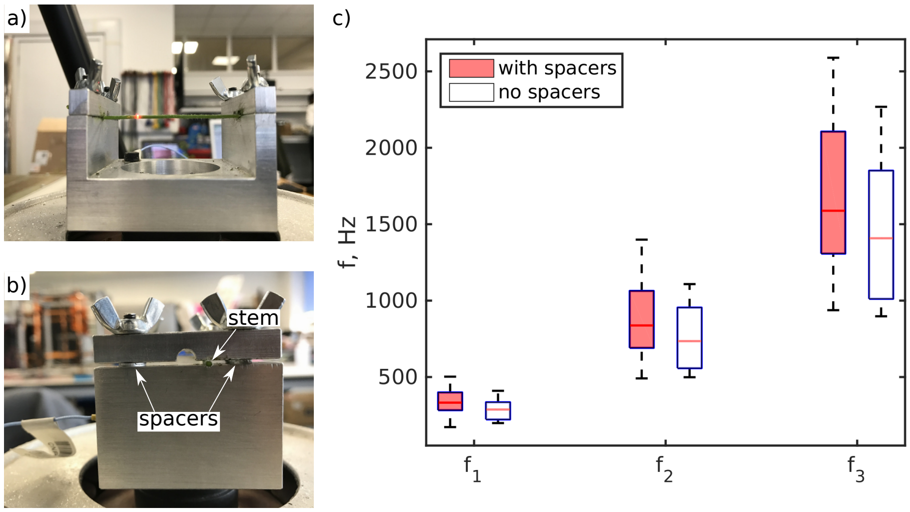


Figure 8: Examination of the effect of clamping in the holder on the measured natural frequencies of Arabidopsis stems. (a) Stem ends are squeezed by the clamps; (b) Spacers are inserted to prevent squeezing of stem ends; (c) Box plots representing the effect of squeezing of stem ends on the natural frequencies of the tested stems (n=25).

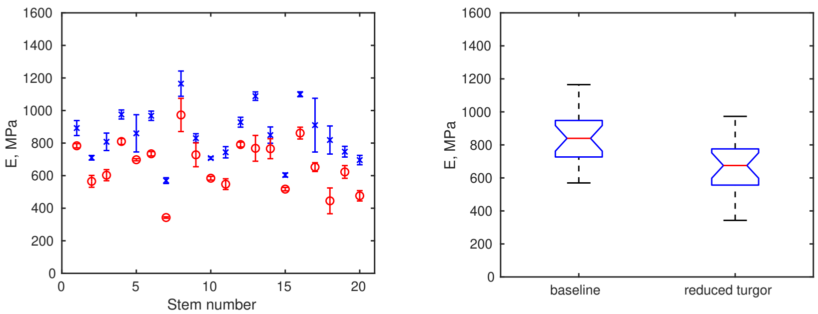
3.3. Influence of stem clamping

The effect of squeezed stem ends on the natural frequencies is presented in Figure 8c. As expected, flattened stem ends reduced all detected natural frequencies of the stems. The observed differences are statistically significant with $p < 0.001$ for the first and $p < 0.0001$ for the second and third natural frequencies. The mean decrease for all three determined frequencies is around 15%. Since for calculation of flexural rigidity from formula (3) f_i should be squared, according to propagation of uncertainty this will result in an error of more than 20% in EI and consequently in E values.

Thus, for vibration tests in a clamped-clamped configuration, attention should be paid to the conditions of the clamped stem ends and the clamping system should be designed in a way that prevents damage. In the current study, all tests were carried out with inserted spacers to mitigate the effect of damaged stem ends on the results.

3.4. Influence of turgor pressure on the mechanical properties of Arabidopsis stems

The mass of the whole tested segment did not show a significant change between the two tests as the observed difference was close to the resolution limit of the precision balance used for its determination. Consequently, for the calculations of the bending rigidity and modulus of elasticity, the mass of the 50 mm segment determined after the second test was used. Also, significant differences were not observed in the values of the stem diameters from both tests. However, taking into account manual image processing that is required to measure the diameter, and some degree of inaccuracy associated with this, the diameter for



(a) (b)

Figure 9: Influence of turgor pressure on the modulus of elasticity of Arabidopsis stems. (a) Mean values of the modulus of elasticity determined for the baseline case (×) and after hyperosmotic treatment (○), errorbars represent standard deviations; (b) Box plot representing E, determined for the baseline tests (n=20) and after hyperosmotic treatment (n=20).

each segment was averaged between two tests and the second moment of area was based on this value. Taking all the aforementioned points into account, all the changes in the mechanical properties of the tested segments of Arabidopsis stems were associated with the decrease in their cell turgor pressure as a result of hyperosmotic stress. With the decrease of the turgor pressure the flexibility will increase, hence the modulus of elasticity is expected to decrease.

After the stems were treated with mannitol, a consistent decrease in their modulus of elasticity was observed in all cases (Figure 9a). This is in line with previous studies on the influence of turgor pressure on the mechanical properties of various plant parts and tissues (e.g. Falk et al. (1958); Faisal et al. (2010)). On average the modulus of elasticity decreased by 185 ± 77 MPa due to the reduction of turgor pressure. Figure 9b shows that this change is statistically significant ($p < 0.0001$). Since the stem diameter remained unchanged the bending rigidity followed the same trend as modulus of elasticity. The decrease in cell turgor pressure made the stems more susceptible to elastic deformations and reduced their resistance to bending through decrease of the modulus of elasticity and bending rigidity respectively.

4. Discussion

4.1. Design of the holder for vibration tests

As any other structure the stem holder for vibration tests has its own natural frequencies that may overlap and interfere with the natural frequencies of the tested stems and consequently affect the results. Thus, the design of the holder must ensure that its natural frequencies are not within the frequency range of interest for the planned tests.

Finite element modelling (FEM) could be used during the design stage of the holder to establish its natural frequencies. Existing holders can be tested separately from the plant stems to determine their suitability.

The holder used in the present study is made of aluminium, and the distance between supports is 50 mm. Based on the results obtained in FEM modelling software, Abaqus (Dassault Systemes, France), the base of the holder was made approximately 2.5 times thicker than the side walls. This aspect ratio results in its first natural frequency being over 6500 Hz. An aspect ratio of 1:1 between the thickness of the base and the wall would lower the first natural frequency to 2800 Hz, hence affecting the vibration tests. Preliminary vibration tests of the holder in isolation confirmed that no peaks are present within testing range (0-4000 Hz).

4.2. Clamped-clamped boundary conditions

Although it is more intuitive to tests plant stems in a clamped-free (cantilever) configuration, since this is how most plants grow in nature, in this study clamped-clamped boundary conditions were used. Clamped-clamped boundary conditions reduce maximum deflection (Blevins, 1979) thus leading to a more linear response, and therefore avoiding modal-coupling energy transfer (Hill et al., 2015). In addition, compared to a cantilever, a clamped-clamped configuration gives more control over the measurement system thanks to the aforementioned lower deformation levels and the existence of at least two nodes with zero displacement in fixed locations along the structure, namely the extremities. This allows for the use of a laser vibrometer which offers an inherently high resolution and sampling rate but also requires that the motion occurs along the direction of the laser beam. The nodes permit to identify two neighbourhoods where the displacements are sufficiently small so that the target movements can be kept perpendicular to the laser beam.

The perfect implementation of any boundary condition is very difficult to achieve in an experimental setup and some degree of uncertainty will be introduced due to this fact. For example, if some degree of flexing was allowed at one or both clamping points, the boundary condition would correspond to clamped-pinned or pinned-pinned respectively. In this case, for the calculation of the mechanical properties of the tested stem segments, values of the parameter λ that correspond to these boundary conditions should be used in equation 3. However, in our current experimental setup we closely approximate clamped-clamped boundary condition as demonstrated by the successful validation against the three-point bending test. Use of λ-values corresponding to clamped-pinned or pinned-pinned boundary conditions would result in a significant mismatch to three-point bending test results.

4.3. Comparison with other recently developed dynamic methods

Recently two other vibration-based methods for characterisation of plants have been developed, namely the methods described in Nakata et al. (2018) and de Langre et al. (2019). In the following section we give a comparison of these methods with the current approach that is summarised in Table 4.

de Langre et al. (2019) presented a method that allows to measure the first natural frequency of free vibration of a whole plant. In addition, it gives the

Table 4: Comparison of the current method with other recent dynamic methods that can be applied on Arabidopsis inflorescence stems.

Parameters	present method	Nakata et al. (2018)	de Langre et al. (2019)
determined frequencies	n natural frequencies $n \in [1..4]^*$	1^{st} natural frequency	1^{st} natural frequency
type of vibrations	forced	free	free
application	mechanical characterisation phenotyping	mechanical characterisation phenotyping	phenotyping
validation	yes	no	n/a
non-destructive	no	no	yes
measured quantities	f_i, EI, E	f_1, EI	f_1

* depending on the mechanical characteristics of the stem

possibility to determine the frequency of each stem in case of multi stem plants. The method is non-destructive, allowing to examine the same plant over a period of time as it grows. The primary purpose of this method is phenotyping of various plants including Arabidopsis. Theoretically, this approach could be extended to the mechanical characterisation of plants, provided the mass of the plant can be measured. As pointed out by the authors non-destructive measurement of the mass of a plant with complex structure is challenging.

The method presented by Nakata et al. (2018) was designed primarily for the characterisation of Arabidopsis stems and allows to determine the first natural frequency from free vibration. It can be applied for identification of Arabidopsis mutants (phenotyping) with altered cell wall properties. In addition, it provides data on bending rigidity of tested stems, and as it is a destructive method it could potentially be extended to the measurement of the modulus of elasticity. However, its capabilities for the mechanical characterisation still require validation against a standard testing method such as the three-point bending test to establish its reliability.

In contrast to the aforementioned methods, the method developed in this study uses a forced vibration approach and provides data on multiple resonant frequencies of the Arabidopsis stems. This data is used for determination of their bending rigidity and modulus of elasticity. Utilisation of several frequencies for the calculation of the mechanical properties increases the overall accuracy of the results. In addition, the presented method was validated against the well-established three-point bending method. While the main purpose of the developed method is to measure mechanical properties, it could also be utilised to identify Arabidopsis mutants with different cell wall properties by measuring their natural frequencies.

4.4. Limitations

Despite a number of advantages, the developed method has also some limitations. As discussed previously, a carefully designed holder is required for clamping of the biological specimens. Attention should also be paid to the design of the clamping system as well as the clamping process itself, as damage to the specimen during clamping can affect the results. Proper clamping of the tested specimen with utilisation of spacers requires training of the operator. This can be considered as a drawback of the presented method compared to the static bending tests, where clamping is not involved.

4.5. Considerations for future studies

The presented method can be used for characterisation of mechanical properties of Arabidopsis stems and changes in them associated with thigmomorphogenesis caused by growth under different conditions mimicking challenging environments (e.g. with mechanical perturbation or subjected to wind). In addition, this method can be utilised for mechanical characterisation of Arabidopsis mutants with altered properties (e.g. mutant without secondary cell walls) and other plants. When applied to other plants and plant parts (e.g. roots), it should be taken into account that values of the natural frequencies depend on a number of parameters (see formula 3). For testing of plant parts that have similar diameter as tested Arabidopsis stems the current setup could be used in the present configuration. However, the number of detected frequencies can be reduced in case tested specimen has lower mass per unit length value or higher modulus of elasticity. A possible solution is to increase the resolution of the system to detect frequencies over a wider range. Stems of larger plants, such as wheat or rice, would require and appropriate scaling of the holder taking into account aforementioned considerations for the holder design and span-to-depth ratio limitations in order for Euler-Bernoulli beam theory to remain valid. Furthermore, when used without immediate frequency data post-processing, it can be applied as a high-throughput technique similar to the one presented by Nakata et al. (2018). The advantage of the current method is that it gives more than one natural frequency and consequently more information on tested plant is available.

Another advantage of the clamped-clamped boundary condition is that it allows to isolate the part of the stem under consideration for testing. Consequently, it would be attractive to evolve the described method into a non-destructive testing technique. However, the main challenge will be to develop a clamping system that minimises stem bruising, since damage to the plant stem will affect plant growth and properties after testing.

5. Conclusions

A new type of multiple resonant frequency dynamic testing method for characterisation of mechanical properties of the inflorescence stems of the model plant *Arabidopsis thaliana* was developed. This method enables the assessment of the bending rigidity as well as modulus of elasticity of the stems. The values obtained by this method were compared to those obtained from more conventional three-point bending tests. For the bottom part of the stems, both methods show a good agreement in the modulus of elasticity values. However, for the top part of the stems the discrepancies between both methods are observed. The presented dynamic method gives the possibility to investigate mechanical properties for a top part of the stem that is more flexible in a more reliable way compared to the three-point bending test. The current samples show approximately 50% of the value of the elastic modulus of the bottom parts. In addition, the overall accuracy of the presented method is higher since more than one natural frequency is obtained simultaneously from each test. The possibility to test different parts of the stem gives an advantage to assess the variation of the properties along the stem instead of obtaining the net value. This will provide a better and more accurate understanding of plant material properties and their changes due to various factors such as mechanical stimuli, drought, etc. that are important for breeding of new plants of high resilience against challenging environmental conditions. Furthermore, one of the potential applications of the developed dynamic method was demonstrated through assessment of changes in the mechanical properties of Arabidopsis stems due to variation in their cell turgor pressure. These tests showed a consistent reduction of the modulus of elasticity and bending rigidity with decrease of turgor pressure.

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