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Mechanical rubbing of tomato internode influence stem growth, improve tensile strength but negatively impact flavonoid levels

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JMN conceived the experiments and SS conducted the experiments. JMN and SS contributed to the analysis, interpretation and writing of manuscript.

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Abstract: Agricultural crops are exposed to different environmental stress factors on a daily basis. Mechanical induced stress (MIS) caused due to contact rubbing, bending, transplantation and spraying of water results in altered growth in plants (Thigmomorphogenesis). The present study was conducted by inducing mechanical stress (gentle rubbing) on the third internode of tomato (*Lycopersicon esculentum* Mill.) by pressing with thumb and index finger (30 sec) for 14 consecutive days. At the end of the stress period, marked morphological differences included significant reduction in plant height and decreased internodal length of the rubbed third internode as well as the neighboring fourth internode. Histochemical staining of the stem cross section of stressed plants showed intense color indicating lignin deposition. Study of biochemical response post internode stress showed an increase in total phenol content but lower flavonoid contents. Stress induction also resulted in modification of biomechanical characteristics like tensile strength, elastic modulus and breaking force. Studies on the effect of mechanical perturbations in plants has gained attention because of its implication in fundamental processes of organogenesis/morphogenesis and their potential as an innovative means of controlling plant growth.

1. Introduction

In the natural habitat, plants are challenged by various biotic and abiotic stress factors. Mechanical Induced Stress (MIS) is one such form of stress that occurs inevitably in plants on a daily basis. MIS is a term used in cases of either a damage or physical injury due to bending, shaking and rubbing experienced by plants as a result of wind exposure, rain, animal movement and agricultural practices (Biddington, 1986). Being sessile, plants do not possess the luxury of relocating from the stressful source and are persistently challenged by these unfavorable environmental conditions. Plants are also known to sense the force of stimuli that can range from subtle effects like touching or rubbing on leaves to more intense

ones like the damage caused by herbivores (Chehab *et al*., 2009). Morphogenic responses due to rubbing or touch are termed Thigmomorphogenesis (Jaffe, 1973). Among the most visible morphological thigmomorphogenesis response common to many plant species includes decrease in shoot elongation, coupled with increased radial dimension of cortex cell (Telewski and Jaffe, 1986; Braam, 2005; Potocka and Szymanowska-Pulka, 2018). Plant cell wall is the first barrier against external mechanical forces. Lignin is one of the main structural components of the plant cell wall and regulation of lignin metabolism is of prime importance to plant growth (Zheng *et al*., 2017). Apart from lignin, flavonoids are among the most crucial phenolic compounds involved in various protective roles in plants, such as attractants, feeding deterrents and even, oxidative stress protection (Treutter, 2005; Mierziak *et al*., 2014). While, stress induced biochemical changes in lignin are known, effect of mechanical stimulation on flavonoid levels are scarce.

Regulation of plant growth to achieve essential agronomic traits such as crop yield and plant height, are important prerequisites for agricultural industries. Marketability of many important ornamental and horticultural crops increases with compact height and a strong stem as these facilitates packaging and also, transportation of post-harvest produce (Babalar *et al*., 2016; Bornke and Rochsch, 2018). The knowledge on force response of stem structures is critical as it incorporates approaches to test biomechanical properties involving tensile strength and elastic modulus. These tests besides having economic implication, is also an immense tool for crop improvement (Shah *et al*., 2017). Tomato, (*Lycopersicon esculentum* Mill.) is one of the most cultivated vegetable crops belonging to *Solanaceae* family (Schwarz *et al.*, 2014). Global tomato production accounts for 170 million tonnes, comprising 75% fresh and 25% processed material for industrial uses (Costa and Heuvelink, 2018). Due to its short duration and relative ease of cultivation, tomato production has become one of the principal horticultural industries (Costa and Heuvelink, 2018).

In this study, we experimentally determined whether stress on the internode by rubbing can lead to thigmomorphogenetic effects, leading to mechanical stress resistance in tomato. It is hypothesized that lignin accumulation due to mechanical perturbations positively regulates higher tensile strength in plant stem. Therefore, the purpose of this study was to establish physiological effects of mechanical stimulation of the 3rd internode on tomato stem growth characteristics and consequently, its mechanical properties in plants.

2. Materials and Methods

Plant material and growth condition

Seeds of tomato (*Solanum lycopersicum* Mill.) belonging to a semi determinate variety (*Solanum lycopersicum* Mill. cv. Arka Vikas) were obtained from Indian Institute of Horticultural Research (IIHR, Bengaluru, India). Germinated seedlings were transplanted to fresh pots (one plant per pot) in green house (12°56‘7” N, 77°35‘3” E) and maintained in moist soil under natural light condition (14 hrs of light and 10 hrs of dark) at 27-28°C. Relative humidity was maintained around 61-70% in the green house. All the experiments were conducted between 10.00 AM - 2.00 PM to avoid early and late diurnal response.

Application of mechanical stimulation

Mechanical stress stimulus was applied to five week old plants, henceforth referred to as internode stress. Internode stress was applied by rubbing the 3rd internode for 14 days, using thumb and index finger for 30s (Depege *et al*., 1997). Plant growth parameters like height and internode length were measured after the application of internode stress for 14 days. The mean total height and internodal length (3rd and 4th internode) of 20 plants were recorded and compared with control plants.

Total phenolic compound content

Total phenolic content in leaves of plants after application of stress were determined by Folin-Ciocalteu method as described by Marinova *et al*. (2005). About 1 gram of fresh leaf was collected by excising the third leaf through random selection from the 20 pots of both control and stressed plants. Evaluation of phenol content was made for each of the independent triplicates (n=3) from 1g of pooled leaf samples for both control and treated plants. Each sample was macerated in 25 ml of methanol for 24 hr with occasional shaking. 250 µl of the extracted solution was mixed with 750 µl of methanol and 1mL of Folin-Ciocalteu reagent was added. After 5 min, 1 mL of Na_2CO_3 (20%) was mixed to the solution. After 30 min of incubation in dark, absorbance was measured at 765 nm using a UV-Vis Spectrophotometer (Shimadzu Inc. Japan) against a blank sample. A stan-

dard Gallic acid curve was constructed by preparing dilutions of a standard solution of Gallic acid. Total phenol was calculated and was expressed as Gallic Acid Equivalents per gram of fresh weight (GAE g^{-1} of FW).

Total flavonoid content

Aluminium chloride calorimetric method was followed for the determination of the total flavonoid content in leaf samples based on the methodology described by Dewanto *et al*. (2002). About 1 gram of fresh leaf was collected by excising the third leaf through random selection from the 20 pots of both control and stressed plants. Evaluation of flavonoid content was made for each of the independent triplicates (n=3) from 1 g of pooled leaf samples for both control and treated plants. Each sample was macerated with 70% methanol and kept for 24 hours with occasional shaking. The extracted sample solution (250 µL) was separately mixed with 75 µL of 5% $NaNO_2$ followed by incubation for 5 min. After incubation, 10% $AlCl_3$ (150 µL) was added followed by 500 µL of 1 M Sodium hydroxide. Total volume was made up to 2.5 ml using distilled water. After the incubation period for 30 min, absorbance was measured at 510 nm against a blank using UV-Vis spectrophotometer (Shimadzu Inc. Japan). Quercetin standard curve was constructed by preparing dilutions of a standard solution of Quercetin for the quantification of total flavonoids. The total flavonoid was calculated and was expressed as quercetin equivalent per gram of fresh weight (QE g^{-1} of FW) (Lin and Tang, 2007).

Lignin staining and quantification analysis

To evaluate how MIS affected lignin deposition, histochemical analysis of the stem sections of control and stressed plants was performed using wiesner reagent. The amount of 2% of fresh phloroglucinol staining solution for lignin staining was prepared in 95% ethanol. Fresh stem sections from the neighbouring 4^{th} internode were immobilized on a sliced potato surface. Multiple homogeneous thin freehand sections were obtained by drawing a sharp razor blade with smooth stokes repeatedly to ensure uniformity. The sections were further checked by stereomicroscope in order to remove thick and also damaged sections. The most uniform sections were picked carefully and transferred to petri plates containing water. Uniform sections of control and treated plants were transferred to petriplates and immersed in phloroglucinol stain. After 24 hours, sections were transferred to a clean glass slide, to which a drop of concentrated HCl was added (Phloroglucinol-HCl stain). The slides were then gently warmed over a Bunsen burner for 20 seconds for HCl evaporation. A drop of glycerol was added to these sections after which a cover slide was placed over it gently. The slides were then observed under a microscope Labomed Vision 2000 (Inc. India) at 10X magnification (Mitra and Loque, 2014).

Lignin quantification analysis was conducted through ImageJ software (ver 1.52p). An eyepiece graticule (scale bar) was used to set the scale measurements for the sections of control and treatment. These images were further processed through a process of spatial resolution information in ImageJ using the calibration set by the graticule image. To start with the quantification analysis, images were duplicated. One of the images was converted from 8 bits to grayscale. Following this action, pixel information pertaining to the pixel colour, gets converted into brightness measurement. By selecting the command: image>adjust>threshold, images to be quantified was highlighted in pixels within the threshold range specified. Next, by choosing the command: analyse>set measurement, the area and limit to threshold was selected. This enabled to measure within the threshold pixel range for control and treated images. Finally, the function, analyze-measure was used to read the area covered with stain. Percentage of stained area was calculated by: Stained area/Total area X 100 (Beziat *et al.,* 2016).

Determination of tensile strength

Determination of biomechanical stem properties was done after 14 days of stress application. Plants of 10 cm length were defoliated after the experiment and five stem segments devoid of leaves and roots were randomly chosen for analysis from stressed and control plants. These stem segments were then kept in an oven (40-50°C) for approximately 1 hour to rid of excessive water content. They were then clamped in the Universal testing machine (MTS-Mechatronics, Inc, Ichalkaranji, India). The following biomechanical traits were determined (Shah *et al*., 2017):

(1) Tensile strength (MPa) is a measure of the maximum stress that the material can withstand without being elongated or stretched.

(2) Elastic modulus (MPa) represents a ratio of stress applied and strain, the material exhibits (degree to which the material resists deformation in response to an applied force).

(3) Breaking force (N) measures the maximum force that the material can bear before its experiences mechanical stress failure.

Statistical analysis

Statistical analysis for the results obtained were carried out using Student's *t* test with level of significance set at P<0.05. Coefficient of variation (CV) is a useful measure to determine dispersion in a variable and can be extremely informative to study variation in different phenotypic traits in a study. Coefficient of variation for the morphological traits obtained was calculated as follows:

(Standard deviation/Average) x 100.

Pearson correlation coefficient was performed to study the level of association between two variables, pair-wise. All these analysis was performed using GraphPad Prism (version.8).

3. Results

Effect of internode stress on plant height and internodal length of tomato

Application of mild mechanical stimuli in the internodes of treated plants resulted in reduction in plant height whereas control plants showed normal growth characteristics (Fig. 1). Internode stress led to 27.4% reduction of total plant height when compared to control plants, a significant reduction at P<0.0001 (Fig. 2 a). Effect of internode stress affected the elongation of 3rd internode, the site of stress application. Interestingly, internode stress also resulted in significant difference in elongation of the neighbouring 4th internode. Results from the present finding showed that elongation of stressed internode was inhibited by 47% and 44% for 3rd and 4th internode respectively at P<0.0001 in comparison to control plants that were not stressed (Fig. 2 b).

Coefficient of variation (CV) studies

The results presented in Table 1 revealed that the mean plant height in control plants was 27.5 cm with a range between 25.2-30.5 cm. In contrast, treatments resulted in mean plant height of 20.01 with range between 18.0-22.8 cm. In case of treated plants, the highest CV was observed for 3rd internode (16.64%), the site of mechanical stimuli followed by the 4th internode (14.8%). In comparison, in control conditions, coefficient of variation (CV) of 5-7% was observed for the traits measured, confirming lower variation within population. Clearly, internode stress

Fig. 1 - *Lycopersicon esculentum* Mill cv. Arka vikas after 14 days of application of mechanical stimuli by rubbing the 3rd internode. Left, control plants and right, treated plants.

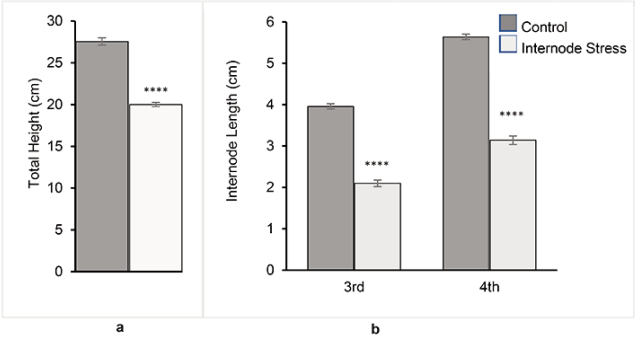

Fig. 2 - Total height of control and internode stressed plants (a) and length of third and fourth internode of control and internode stressed plants (b). The vertical bars indicate standard errors. The values correspond to the mean ± SE, where n=20. Statistical analysis was performed by Student's t-test for total height and internode length. Asterisks indicate mean values significantly differ at P<0.05 (**** indicate P <0.0001).

Table 1 - Range of group means, mean values, standard deviation, standard error mean and coefficient of variation of internode length and total height of control and treated plants

Traits	Control					Treatment				
	Range	Mean	SDV	SEM	CV (%)	Range	Mean	SDV	SEM	CV (%)
3rd Internode	3.6-4.5	3.9	0.287	0.064	7.3	1.5-2.6	2.1	0.349	0.078	16.64
4th Internode	5.2-6.3	5.6	0.298	0.066	5.3	2.5-3.8	3.1	0.46	0.103	14.8
Total Height	25.2-30.5	27.5	1.9	0.429	6.9	18-22.8	20.01	1.097	0.245	5.48

* Values in the table mentioned for n=20. Descriptive statistics for control and treated plants SDV= Standard deviation; SEM= Standard error of mean; CV= Coefficient of variation.

lead to higher variability for the 3rd and 4th internode than in normal conditions as indicated by these results.

Effect of internode stress on total phenol and flavonoid content

Mechanical stimuli resulted in an increase of 25% total phenolic content in leaves, when compared to control plants, a significant increase at P = 0.0001 (Fig. 3 a). In contrast, flavonoid content in leaves of treated plants showed a reduction of 18% in comparison to control plants, a significant decrease at P = 0.0013 (Fig. 3 b).

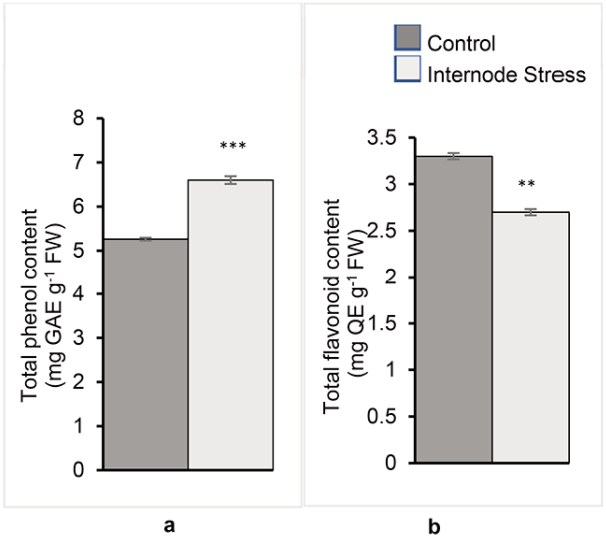

Fig. 3 - Total phenol content expressed as mg of gallic acid equivalent per gram of fresh weight (GAE g^{-1} FW) after 48hrs of internode stress (a) and total flavonoid content expressed as mg of Quercetin Equivalent per gram of fresh weight (QE g^{-1} FW) after 48hrs of internode stress (b). Standard error is indicated by vertical bars. The values correspond to the Mean ± SE, where n=3. Statistical analysis was performed by Student's t test. Asterisks indicate Mean values significantly differ from control at P <0.05 (** indicate P = 0.0013 and *** indicate P = 0.0001).

Effect of internode stress on lignin deposition and tensile strength

Histochemical analysis by staining revealed that the intensity of colour was visibly lower in control plants (Fig. 4 a). On the other hand, analysis revealed intense colouration (red/magenta) in stressed plants (4th internode) indicating increased lignin deposition in xylem bundles and confirming alteration in lignin deposition in the plants with internode stress (Fig. 4 b). Lignin quantification through ImageJ analysis revealed a 45% enhancement in lignified area for sections from treated plants. On the other hand, sections from control samples covered only 22% of lignified area (Fig. 4 c).

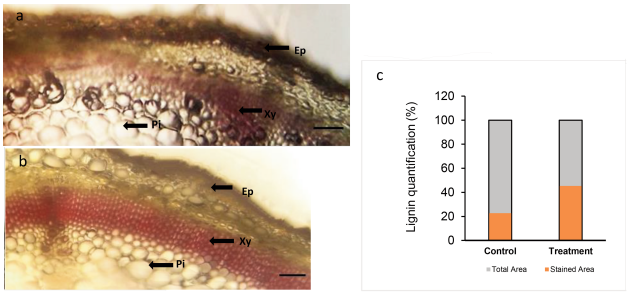

Fig. 4 - Microscopic view of stained cross sections after 14 days of rubbing of the third internode for control (a) internode stressed (b) and graph showing quantification of lignin analysis by ImageJ software (c). Ep-epidermis, Xy- xylem (lignin deposition) and Pi-pith. Scale bar = 100 µm.

Application of mechanical stimuli also led to increased mechanical strength as indicated by the various mechanical properties shown in figure 5. The mean tensile strength recorded a significant increase of 113% in treated plants, when compared to control plants at P<0.05 (Fig. 5 a). Other mechanical properties including elastic modulus, and breaking force were also significantly higher when compared to the control plants. The elastic modulus displayed by treated plants observed was 658.4 MPa, a highly significant increase (P<0.0001) compared to control plants (Fig. 5 b). The mean breaking force for treated plants recorded was 21.13 N, in comparison to control plants, which was lower (9.8 N), a significant difference at P= 0.0007 (Fig. 5 c).

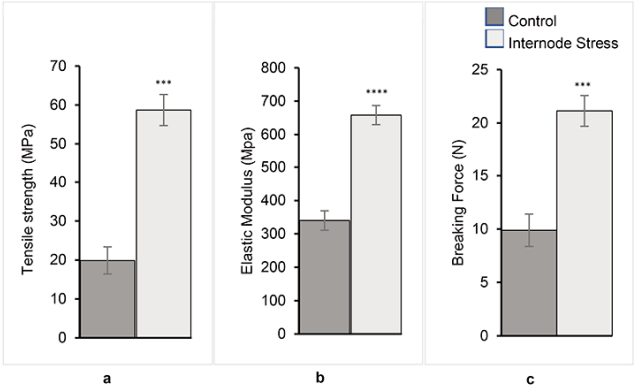

Fig. 5 - Biomechanical properties of the stem measured through tensile test: tensile strength (a), elastic modulus (b) and breaking force (c). Standard errors are indicated by vertical bars. The values correspond to the Mean ± SE, where n=5. Asterisks indicate Mean values significantly differ at P<0.05 (*** indicate P=0.0007 and **** indicate P<0.0001).

Correlation studies among biomechanical trait

The correlation coefficient among tensile strength, breaking force and plant height for both control and treated plants are reported in Table 2. Plants treated with internode stress showed a highly significant positive correlation (R = 0.999) among the two important mechanical properties for stem, tensile strength and breaking force. However, the correlation for these traits in control plants remained non-significant. The correlation of total height of plant with respect to tensile strength and breaking force demonstrated a significant negative correlation for both treatment and control plants. Plants treated with internode stress showed a slightly higher negative correlation (R= -0.97) than control plants (R= -0.91) between tensile strength and total height. A scatter plot of these associated traits clearly highlighted a negative slope of correlation after exposure to mechanical stimuli (Fig. 6).

Table 2 - Linear correlation coefficient between different biomechanical characters in control and treated plants. Correlation of morphological trait with biomechanical characteristics

Traits	Total plant height	Breaking force
Control		
Tensile Strength	-0.91 *	0.7 NS
Total Plant Height		-0.94 *
Treated plants		
Tensile Strength	-0.97 **	0.999 ****
Total Plant Height		-0.95 **

Asterisk indicates statistical significance, at P=0.12(NS), 0.033(*), 0.002(**) and <0.0001(****), NS= non significance difference.

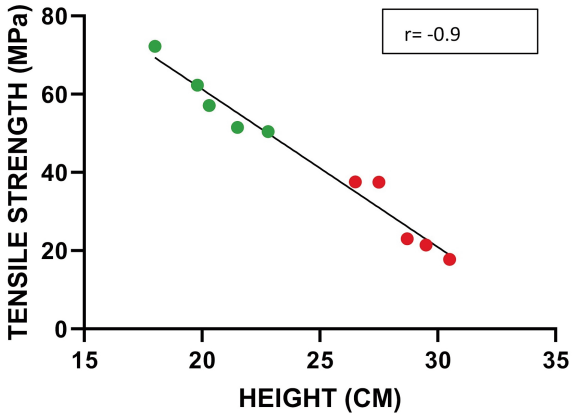

Fig. 6 - Scatter plots of tensile strength and total height measured on treated plants (green) and control plants (red). The scatter plot indicates a significant negative correlation between two variables with a correlation coefficient of r=-0.9, (P<0.0001).

4. Discussion and Conclusions

In response to mechanical stimuli, plants respond by altering their growth rate and morphology (Chehab *et al*., 2009). In this communication, application of internode stress was shown to be effective in reducing the overall height of the plant. Effect of brushing in reducing leaf size and stem elongation has been demonstrated in *Lycopersicon esculentum* (Mill.) (Johjima *et al*., 1992). In *Arabidopsis thaliana* (Linn.), inhibition of stem elongation in the inflorescence was also shown to be prominently affected due to mechanical perturbation (Victor and Rowe, 2011). A higher coefficient of variation in treated plants shown in this study demonstrated that the most relevant effect of internode stress treatment was to bring growth alteration by causing reduction in internode elongation for both the 3rd and the 4th internode. In comparison to the treatment, coefficient of variation in control plants was lower, revealing insignificant random variation within a phenotypic trait. Hence, reduction in internodal length clearly indicates that mechanical stress response is a generalized response affecting both the rubbed internode as well as neighboring young internode. Plants do not possess specialized cells to mount a response to Mechanical Induced Stimuli (MIS) in nature (Börnke and Rocksch, 2018). Individual cells in the vicinity of local tissue that is mechanically stimulated may possess the property of mechanoperception and to transduce this signal to an unperturbed distal tissue either by hormonal or electrical signals (Erner *et al*., 1980). Depege *et al*., (1997) suggested that the transduction of the signal was electrical occurring through the involvement of intercellular Ca^{2+} modulated by calmodulin gene expression. Moulia *et al*. (2015) further proposed that local mechanosensing and the origin of ionic currents can be explained through the participation of mechanosensitive ionic channels or stretch-activated channels (SAC).

Under our experimental conditions, while the phenolic contents increased, flavonoid content was reduced by application of mechanical stress. In plants, the phenylpropanoid metabolism and also the lignin biosynthetic pathway are stimulated by environmental stress adaptation (Petersen *et al.,* 1999). Saidi *et al*. (2009) reported an increase of all the major enzymes involved in the phenylpropanoid pathway with mechanical stimulation in tomato. Consequently, an increase in phenolic content and

enrichment of lignin observed in this study is consistent with these reports. However, biosynthesis of lignin pathway and flavonoid are highly co-regulated in plants, where repression of one pathway, redirects the metabolites into the other pathway (Besseau *et al*., 2007). In the phenylpropanoid pathway, *p*-coumaroyl CoA is the branch point in the metabolic route leading towards biosynthesis of either one of them (Yeh *et al*., 2014). In plants, accumulation of flavonoid is affected as the common flavonoid-lignin pathway is diverted towards utilization of support mechanism for biosynthesis of lignin (Besseau *et al*., 2007). A reduction in flavonoid content observed in this study therefore, points to the proposition that lignin and flavonoid may be competing for the same substrates leading to a decrease in flavonoid synthesis.

MIS increased stem resistance to tensile forces in this study. The modification in these parameters is relatively fast as the experiment lasted for duration of only 14 days. In plants, important biomechanical traits for instance, higher resistance to tensile and breaking forces leads to the acquisition of a hardened phenotype (Schoelynck *et al*., 2015; Shah *et al*., 2017). Cell wall rigidification due to accelerated lignification was shown to result in reduced internodal elongation after mechanical stress application in tomato (Saidi *et al*., 2009). Further, reinforcement of cellulose microfibrils by lignins was also reported to increase resistance to tensile forces (Genet *et al*., 2005). This unique thigmomorphogenetic response in plants is crucial to withstand repeated external stress and mechanical forces (Jaffe *et al*., 1984; Biddington, 1986). Moreover, plant height was shown to be negatively correlated with tensile strength and breaking force in this study. Plant lodging, an important trait observed in plants is shown to occur as a consequence of permanent displacement of plant stem by stem buckling (Kendall *et al*., 2017). Reduction in plant height and internodal length has been shown to improve lodging tolerance in crops, an important trait for crop improvement (Peng *et al*., 2014). Notably, introduction of dwarfing genes was an important outcome of the Green revolution, reducing lodging susceptibility by reducing plant height (Spielmeyer *et al*., 2002).

In conclusion, we observed that besides modifying growth, mechanical stimulation due to rubbing resulted in increased lignin deposition and also enhanced tensile strength leading to overall gain in the physical strength of the plant stem. Further, our results provide new insights on the lignin-flavonoid pathway after MIS and demonstrate the plasticity of the biochemical response. The ability to sense and respond to mechanical stimulation by thigmomorphogenesis needs to be explored, as it holds great potential for commercial application in horticulture for regulating plant growth without the use of chemical regulators (Börnke and Rocksch, 2018).

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