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**ARTICLE**

# Biochemical and Physiological Responses of *Arabidopsis thaliana* Leaves to Moderate Mechanical Stimulation

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**ABSTRACT**

Mechanical stimulation of plants can be caused by various abiotic and biotic environmental factors. Apart from the negative consequences, it can also cause positive changes, such as acclimatization of plants to stress conditions. Therefore, it is necessary to study the physiological and biochemical mechanisms underlying the response of plants to mechanical stimulation. Our aim was to evaluate the response of model plant *Arabidopsis thaliana* to a moderate force of 5 N (newton) for 20 s, which could be compared with the pressure caused by animal movement and weather conditions such as heavy rain. Mechanically stimulated leaves were sampled 1 h after exposure and after a recovery period of 20 h. To study a possible systemic response, unstimulated leaves of treated plants were collected 20 h after exposure alongside the stimulated leaves from the same plants. The effect of stimulation was assessed by measuring oxidative stress parameters, antioxidant enzymes activity, total phenolics, and photosynthetic performance. Stimulated leaves showed increased lipid peroxidation 1 h after treatment and increased superoxide dismutase activity and phenolic oxidation rate after a 20-h recovery period. Considering photosynthetic performance after the 20-h recovery period, the effective quantum yield of the photosystem II was lower in the stimulated leaves, whereas photochemical quenching was lower in the unstimulated leaves of the treated plants. Nonphotochemical quenching was lower in the stimulated leaves 1 h after treatment. Our study suggested that plants sensed moderate force, but it did not induce pronounced change in metabolism or photosynthetic performance. Principal component analysis distinguished three groups–leaves of untreated plants, leaves analysed 1 h after stimulation, while stimulated and unstimulated leaves of treated plants analysed 20 h after treatment formed together the third group. Observed grouping of stimulated and unstimulated leaves of treated plants could indicate signal transduction from the stimulated to distant leaves, that is, a systemic response to a local application of mechanical stimuli.

**KEYWORDS**

*Arabidopsis thaliana*; mechanical stimulation; oxidative stress; antioxidant enzymes; photosynthetic performance; systemic response

## 1 Introduction

In both natural habitats and agricultural fields, plants are exposed to fluctuating environmental factors that are considered as stressful due to their negative effects on plant growth, development, and productivity. Stress factors can be biotic or abiotic. While biotic factors include plant interactions with other organisms, such as herbivore attack and pathogen infection, abiotic factors include environmental cues such as water and nutrient availability, temperature, and light intensity [1–3].

Plants also sense and respond to a variety of mechanical stimuli to which they may be exposed in nature. Considering the widespread effects on growth and development, plants respond to mechanical stress in different ways. It has been shown that the forces originated from touching inhibit growth and delay flowering in *Arabidopsis thaliana* (L.) Heynh. and can reduce the height of cotton plants but did not significantly affect cotton flowering and yield [4–9]. In the sensitive plant *Mimosa pudica*, the carnivorous plant Venus flytrap *Dionaea muscipula*, and sundews of the genus *Drosera*, touch causes distinct and very rapid movement of certain organs [10,11]. In addition, environmental conditions such as strong winds, frost, rain, hail, flooding, soil movement, trampling by animals, and various human activities cause mechanical stress to plants that can lead to bending, rubbing, movement of organs, or damage and even breakage of plant parts.

Acclimation of plants to disturbances caused by mechanical stimuli is based on developmental responses that modulate the mechanical properties of exposed tissues and organs [4,11–13]. It was shown that *A. thaliana* acclimates to mechanical stimulation in the form of brushing by inhibiting the length of the inflorescence stem and altering its mechanical properties [14,15]. When plants successfully recover from mechanical stress, they can maintain agronomically beneficial characteristics. In this context, Iida [5] described a method called "Mugifumi" that has been used by Japanese farmers for centuries. The method is based on empirical knowledge about the beneficial effects of mechanical stress. Wheat and barley sown in the fall were subjected to treading in winter period, which induced morphological changes such as strengthening of roots and shortening of plant height, resulting in higher grain yields. Nowadays, mechanical stress is the focus of much research that continues to expand our knowledge of morphological changes in plants exposed to mechanical stimuli [6,9,11,15–17]. Moreover, mechanically-induced stress can increase the content of specialized metabolites and antioxidant capacity of leafy vegetables [13]. It has also been reported [18] that mechanical stimulation can improve the nutritional properties of plants by increasing the absorption of elements and changes in metabolite profile. In addition, mechanical stimulation has been shown to lead to acclimation and tolerance to other stress conditions such as frost, cold, heat, salt, drought, and pathogen attack [19,20]. An example is the resistance of *A. thaliana* to the fungal pathogen *Botrytis cinerea* after exposure to mild mechanical stimulation [21,22].

The perception of mechanical stimuli begins at the cellular and tissue levels with the activation of specific signalling pathways. Induced molecular and physiological responses include alteration of enzyme activities, degradation of proteins, lipid peroxidation in the biological membranes, alteration of gene expression and *de novo* protein synthesis, accumulation of stress protective metabolites such as compatible osmolytes and various specialised metabolites, and changes in carbohydrate metabolism including modulation of photosynthetic activity [19,23–29]. In addition to local responses to stressors triggered at the site of exposure, certain environmental factors, particularly biotic stresses such as pathogen and herbivore attack, can induce a systemic response that ensures an effective stress response in tissues distant from the site of the initial attack [30–33]. The induction of a systemic response by mechanical stimulation or injury has been reported in several studies and is characterised by the accumulation of jasmonate, a well-characterised signalling molecule, and increased accumulation of ROS, followed by $Ca^{2+}$ influx [34–37].

A common response to most stress factors, including mechanical stress, is increased production of reactive oxygen species (ROS) that are derivatives of molecular oxygen ($O_2$) such as superoxide radicals, hydroxyl radicals, hydrogen peroxide ($H_2O_2$), and singlet oxygen, and are usually considered highly toxic to the cell [7,38,39]. A certain amount of ROS is regularly produced in cells during metabolic reactions and is involved in many cellular processes, such as cell wall strengthening, senescence, response to wounding and pathogen attack. In addition, ROS also serves as signals in developmental processes such as root growth and cellular differentiation [40], activation of various adaptive mechanisms to stress conditions, and defence against pathogens [41,42]. Apart from these beneficial functions, excessive production of ROS and its strong oxidising activity can seriously disrupt the structure and metabolic function of macromolecules such as proteins, nucleic acids, and membrane lipids [41,43,44]. The level of lipid peroxidation, often measured as malondialdehyde (MDA) content, is an indicator of oxidative damage to membrane lipids [45].

Plants have evolved an efficient antioxidant system consisting of enzymatic and non-enzymatic components distributed in different subcellular compartments, and which together play a role in mitigating the deleterious effects of excessive ROS production [41,43]. The most important antioxidant enzymes in plants are superoxide dismutase (SOD), catalase (CAT), and peroxidases (PODs) such as ascorbate peroxidase (APX) and guaiacol peroxidase (G-POD) [38,46]. The $H_2O_2$ content and activity of antioxidant enzymes are important indicators of plant response to various stimuli. Increased activities of antioxidant enzymes are reported in many studies on the effects of abiotic stress on plants [47,48].

Phenolic compounds, such as flavonoids, tannins, coumarins, lignans, and phenolic acids, play an important role in the interactions between plants and their environment. Phenolics are structural elements of the cell wall, and important components of the defence response to infection, excessive sunlight, injury, and heavy metal stress [49–52]. Therefore, the content of phenolics in plant tissues can vary under the influence of environmental conditions, and their accumulation serves as a good indicator of plant response to a specific stress [53–55]. Polyphenol oxidases (PPOs) are nearly ubiquitous enzymes in plants involved in biosynthetic and cross-linking reactions, as well as defence against abiotic and biotic stress factors [56,57]. No PPO genes have been identified in the genus *Arabidopsis*, and it has been suggested that the absence of PPO in this genus could be compensated by other oxidative enzymes such as laccases and peroxidases that could perform similar phenolic oxidations using $O_2$ [58].

Plant growth and biomass production depend on photosynthesis, a complex process involving numerous cellular components such as photosynthetic pigments and all other elements of the electron transport chain, carbon dioxide assimilation, and biosynthesis of starch and sucrose. Adverse environmental conditions can affect photosynthesis [59,60]. The kinetics of chlorophyll fluorescence induction upon irradiation of dark-adapted leaves is one of the methods used to determine photosynthetic performance. Measured and calculated parameters obtained by this non-destructive and highly sensitive method provide useful information on photosystem II (PSII) efficiency and electron transport in chloroplasts [60–62]. Analysis of photosynthetic pigments in plants–the levels of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids (Cars) as well as their ratios provides a useful tool for assessing the effects of environmental stress on light-absorbing efficiency and photosynthetic capacity [60,63,64].

According to our observations and data in the literature, mechanical stimulation has been less studied than effects of other abiotic and biotic environmental conditions. The studies conducted so far included different types of mechanical stimuli, however they were rarely quantified, making it difficult to compare data from different studies as pointed out by Coutand [65]. In most cases, mechanical stimuli have been quantified as the number of rubbing [22], touching [21], bending [66], and brushing treatments [15], and these studies have focused mainly on the effects on growth, morphological, and developmental changes [18,67]. Unlike these long-term effects, mechanical stimulation triggers numerous physiological and

biochemical changes that can be detected in a much shorter time than phenotypic modifications. Interesting findings describing the generation of ROS, induction of the antioxidant system, hormonal modulation, and activation of gene expression upon mechanical stimulation have been published [7,21–23,68,69], but in general such effects are less studied compared to the previously mentioned morphological changes.

The main objective of our work was to apply an external, precisely quantified, moderate mechanical force of 5 N (newton) to the leaf blades of *A. thaliana*. Based on our personal sense and estimation, the stimuli could be compared to the pressure caused by the movement of animals, such as trampling, and by weather conditions, such as heavy rain. As mentioned earlier, mechanical force can have detrimental effects on plants, but various forms of mechanical stimuli such as wind, touch, brushing, or bending can improve plant defence responses and stress tolerance [22,32,70]. Therefore, mechanical stimulation has a potential for crop production [19,21]. However, for successful application of this method there is a need to understand the physiological and biochemical basis of the response to mechanical stimuli, including the mechanism of signal transduction to distant tissues. To contribute to the existing knowledge on the response to mechanical stimulation, we used a model plant *A. thaliana* to evaluate selected physiological and biochemical changes: oxidative stress parameters, activation of antioxidant enzymes, phenolics, photosynthetic performance, and pigment content. In our experiment, leaves of *A. thaliana* subjected to mechanical stimulation were sampled 1 h after short-term exposure to the mechanical force and after a recovery period of 20 h. To study a possible systemic response, unstimulated leaves of treated plants were collected alongside the stimulated leaves of the same plants 20 h after the treatment.

## 2 Materials and Methods

### 2.1 Plant Material and Growth Conditions

Seeds of *A. thaliana*, ecotype Columbia were sown in square plastic pots (6 cm × 5.5 cm) filled with soil. After watering the soil, the pots were covered with plastic wrap and placed in the dark at 4°C for stratification. After three days, the pots were moved to the growth chamber where the plants were grown at 20°C, relative humidity of ~60%, and a photoperiod of 10 h of light and 14 h of darkness. Illumination was provided by cool white fluorescent tubes (18 W) with a light intensity of 60–65 $\mu mol\ m^{-2}\ s^{-1}$ at plant level. The plastic wrap was removed from the pots as soon as the germinated seedlings became visible. Plants were irrigated twice weekly by filling the trays with water that was absorbed into the soil through the perforated pot bottom.

According to Boyes et al. [71], the stage of the plants used for the experiment was: rosette growth complete (stage 3.90), before flowering, i.e., before the growth of the flower stalk and without a dark green rosette. Leaves reached their final size and mechanical stimulation was applied to the oval shaped leaf blade.

### 2.2 Mechanical Stimulation

Two to three leaves per plant were subjected to moderate mechanical stimulation using a force of 5 N over an area of approximately 1 $cm^2$ (a pressure of approximately 50 kPa) for 20 s. Leaves were pressed on both sides of the leaf blade; the lower side of the blade was pressed with a Force sensor (FlexiForce, model A201, Tekscan), while the upper side with the cap of an Eppendorf tube (its upper, smooth surface) attached to the finger (Fig. 1a). The applied force was measured with a force measurement system (Fig. 1b) and graphically observed in real time on a personal computer [72].
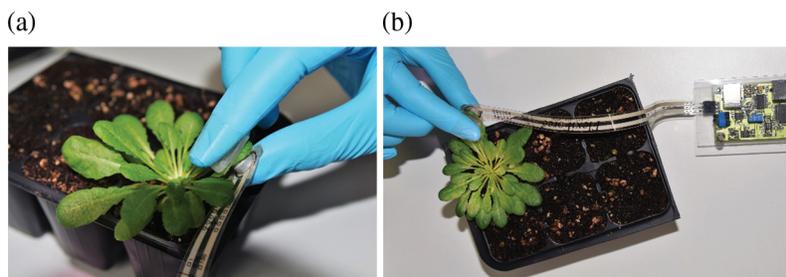
(a)                                                                          (b)



**Figure 1:** Exposure of *A. thaliana* leaves to mechanical stimuli: a force of 5 N was applied to a leaf area of approximately $1.0 \text{ cm}^2$ (a pressure of approximately 50 kPa) for 20 s. Plant leaves were pressed on both sides of the leaf blade (a); for the application of mechanical stimulation a Force sensor FlexiForce, model A201, Tekscan was used (b)

Three groups of plants (with 5 to 6 plants per group) were used for the experiments: (1) control plants that were not subjected to mechanical stimulation, (2) plants subjected to mechanical stimulation from which stimulated leaves were sampled 1 h after stimulation, and (3) plants subjected to mechanical stimulation from which stimulated and unstimulated leaves were sampled 20 h after stimulation.

After collection, leaves were prepared for analysis. Selected parameters analysed in this work were the $H_2O_2$ content, the degree of lipid peroxidation measured as MDA content, the activity of antioxidant enzymes (CAT, G-POD and SOD), the content of total phenolics, the phenolic oxidation rate (POR), the activity of PSII and the content of photosynthetic pigments.

### 2.3 Measurement of $H_2O_2$

The amount of $H_2O_2$ was determined according to the method of Alexieva et al. [73]. Approximately 100 mg of fresh leaf tissue was homogenized in 1.0 mL of ice-cold 0.1% (w/v) trichloroacetic acid (TCA). The extract was centrifuged at $12,000 \times g$ for 15 min at 4°C. For spectrophotometric measurement, 500 µL of supernatant, 500 µL of 10 mM potassium phosphate buffer (pH 7.0), and 1 mL of 1 M potassium iodide (KI) were added to the quartz cuvette, and the absorbance of the mixture was read at 390 nm. The $H_2O_2$ content was calculated using a standard curve and the results were expressed as ng $H_2O_2$ per mg of fresh tissue.

### 2.4 Determination of Lipid Peroxidation Level

The level of lipid peroxidation was expressed as the amount of MDA, a product of lipid peroxidation, according to the modified method of Heath and Packer [74]. Approximately 100 mg of fresh leaf tissue was homogenized in 1,300 µL of 0.25% (w/v) 2-thiobarbituric acid (TBA) in 10% (w/v) TCA. Samples were incubated at 90°C for 30 min, cooled on ice, and centrifuged at $15,000 \times g$ for 10 min at 4°C. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The degree of lipid peroxidation was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nmol MDA per gram of fresh tissue.

### 2.5 Protein Extraction and Measurement of Enzyme Activity

Leaf tissue (approximately 100 mg fresh weight) was homogenized in 1.5 mL of ice-cold 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 3% (w/v) polyvinylpolypyrrolidone (PVPP). Samples were centrifuged at $20,000 \times g$ for 30 min at 4°C. The supernatants were used for spectrophotometric determination of proteins, measurement of SOD, CAT, G-POD activity and POR. Soluble protein content in the extracts was determined according to Bradford [75] using bovine serum albumin as a protein standard.

SOD (EC 1.15.1.1) activity was estimated as inhibition of nitroblue tetrazolium (NBT) reduction according to the method of Beauchamp et al. [76]. The content of superoxide anions converting NBT to a formazan dye was decreased by the activity of SOD in the experimental sample. The enzymatic system xanthine-xanthine oxidase was used to generate superoxide radicals. The rate of formazan formation was measured spectrophotometrically at 560 nm. The standard curve was obtained from linearized absorbance data plotted against SOD concentration using bovine SOD as standard. The SOD activity unit (1 U) was defined as the amount of enzyme that causes 50% inhibition of NBT reduction. Results were expressed in units per mg of protein.

CAT (EC 1.11.1.6) activity was determined according to the method of Aebi [77], which is based on the rate of $H_2O_2$ consumption, and measuring the decrease in absorbance at 240 nm. Catalase activity was calculated using an extinction coefficient of 40 $mM^{-1}$ $cm^{-1}$ and expressed in nmol $H_2O_2$ per min and mg of protein.

G-POD (EC 1.11.1.7) activity was measured as an increase in absorbance at 470 nm as a result of the $H_2O_2$-dependent oxidation of guaiacol to tetraguaiacol, according to Chance et al. [78]. The extinction coefficient used for the calculation was 26.6 $mM^{-1}$ $cm^{-1}$ and the results were expressed as nmol of tetraguaiacol per minute and mg of protein.

POR was monitored according to the method of Escalante-Minakata et al. [79] with slight modifications. The reaction mixture consisted of 200 μL protein extract and 800 μL 0.1 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and 50 mM catechol. Oxidation of catechol was recorded as the increase in absorbance at 420 nm and expressed as the change in absorbance ($\Delta A_{420}$) per minute per mg of protein.

### 2.6 Determination of Total Phenolic Content

The concentration of total phenolic compounds was determined according to the method of Humadi et al. [80] with slight modifications. Lyophilized leaf tissue (approximately 10 mg) was homogenized in 1.0 mL of ice-cold 80% (v/v) methanol. Samples were incubated in a water bath at 65°C for 45 min and then centrifuged at 15,000 × $g$ for 10 min at 4°C. The reaction mixture consisted of 1,550 μL $dH_2O$, 50 μL of the extract, and 100 μL of the Folin-Ciocalteu reagent. After the addition of 300 μL of 1.88 M sodium carbonate ($Na_2CO_3$), the samples were incubated at 45°C for 30 min. The absorbance was measured at 765 nm and compared with the standard curve obtained with solutions of gallic acid. The total phenolic content of the samples was expressed as μg gallic acid equivalents per g of dry tissue.

### 2.7 In Vivo Measurement of Chlorophyll Fluorescence

*In vivo* chlorophyll fluorescence was measured by the saturation pulse method [81] using the Chlorophyll Fluorescence Package (Quibit System Inc., Canada). Prior to measurement, plants were adapted to darkness for 30 min. After recording a fluorescence minimum ($F_0$) using a weak red light (<5 μmol $m^{-2}$ $s^{-1}$), a short duration pulse of high intensity (~5,000 μmol $m^{-2}$ $s^{-1}$) was applied to induce the maximum fluorescence ($F_m$). After quenching of $F_m$ in darkness, leaves were illuminated with continuous actinic light (intensity ~150 μmol $m^{-2}$ $s^{-1}$), and saturation pulses were applied at 20-s intervals. Actinic irradiation was maintained until the values of maximum fluorescence ($F'_m$) and steady-state fluorescence ($F_t$) became stable. At the end of the experiment (after the actinic light was turned off), the minimum fluorescence value ($F'_0$) was measured under red illumination. The parameters measured during the experiment ($F_0$, $F_m$, $F_t$, $F'_m$, $F'_0$) were used to calculate the maximum quantum yield of PSII ($F_v/F_m$), the effective quantum yield of PSII ($\Phi_{PSII}$), photochemical quenching (qP), and non-photochemical quenching (NPQ).

### 2.8 Measurement of Photosynthetic Pigments Content

Lyophilized and powdered leaf tissue (~30 mg) was homogenised in 1.5 mL of ice-cold 95% (v/v) acetone with the addition of ~50 mg calcium carbonate ($CaCO_3$). After centrifugation at $5,000 \times g$ for 10 min at 4°C, the supernatant was stored on ice while the remaining pellet was resuspended in acetone and centrifuged again. The supernatants from both cycles were combined and filled up to a constant volume with acetone. The absorbance of the extracts was measured spectrophotometrically (Specord 40, Analytik Jena) at 470, 646, and 663 nm. The content of photosynthetic pigments (Chl *a*, Chl *b*, and Cars) was calculated according to Wellburn [82] and expressed as µg of pigment per mg of dry tissue.

### 2.9 Statistical Analysis

Data presented are means of five to six independent biological replicates ± standard error (SE). The values obtained were tested for the assumptions of normality and homogeneity of variance. To detect significant differences between groups ($p \leq 0.05$), Kruskal-Wallis test, followed by Dunn's test for non-parametric comparisons was performed. Statistical analysis was performed using the Statistica 13 software package (TIBCO Software Inc.).

Principal component analysis (PCA) was based on data from replicates for four sample groups–untreated leaves from control plants (C), stimulated leaves sampled 1 h after the treatment (1 h), stimulated leaves sampled 20 h after the treatment (20 h), and unstimulated leaves sampled from treated plants 20 h after the treatment (SR 20 h); and 15 variables–activity of three antioxidant enzymes (CAT, SOD, G-POD), POR and phenolic content (PC), $H_2O_2$ and MDA content, as well as fluorescence parameters ($F_v/F_m$, $\Phi_{PSII}$, qP, NPQ), pigment content (Chl *a*, Chl *b*, Cars) and the ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/Chl *b*). PCA analysis was performed using R 3.6.2. software and the "*factoextra*" package.

## 3 Results

To assess the effects of mechanical stimuli on plant metabolism, leaf blades were subjected to a moderate force of 5 N for 20 s. The applied force did not cause visible damage such as chlorosis or necrosis of the stimulated leaves. After treatment, a series of biochemical and physiological parameters have been examined.

### 3.1 $H_2O_2$ Content and Lipid Peroxidation

Mechanical stimulation triggers $H_2O_2$ production and lipid peroxidation in *A. thaliana* leaves. Considering $H_2O_2$ content, there was no significant difference in $H_2O_2$ content between control (untreated) and treated plants. However, $H_2O_2$ content in stimulated leaves sampled 20 h after treatment was significantly increased compared to the content measured 1 h after stimulation (Fig. 2a).

The level of lipid peroxidation, expressed as MDA content, increased in leaf tissue 1 h after mechanical stimulation. Upon recovery, 20 h after the treatment, stimulated and unstimulated leaves showed similar levels of lipid peroxidation as the leaves of the control (untreated) plants (Fig. 2b).

### 3.2 Enzyme Activities

Mechanical stimulation induced activity of antioxidant enzymes and POR. The activity of the antioxidant enzymes SOD, CAT, G-POD and POR showed a similar pattern in response to mechanical stimulation (Fig. 3). Enzyme activity was low in the control (untreated) plants. After mechanical stimulation, the activity of SOD and CAT almost doubled 1 h after the treatment and remained at the same level 20 h after the treatment (Figs. 3a and 3b). The activity of G-POD and POR gradually increased after mechanical stimulation (Figs. 3c and 3d). However, for CAT and G-POD the differences between the studied plant groups were not statistically significant (Figs. 3b and 3c).
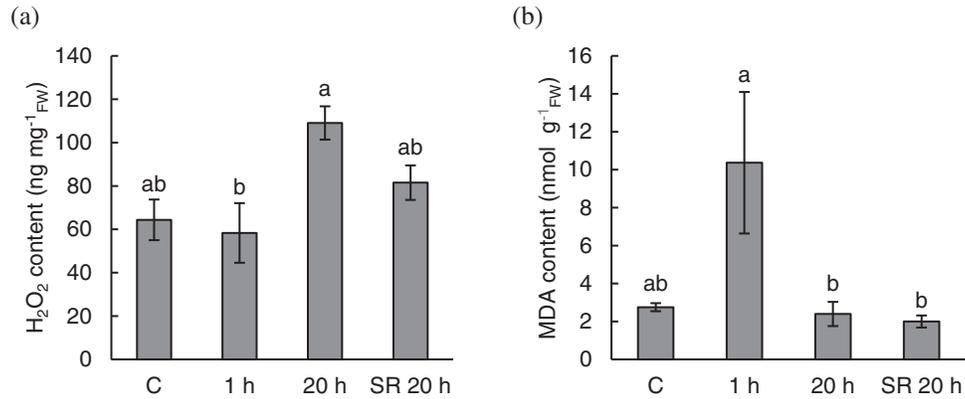
**Figure 2:** Hydrogen peroxide ($H_2O_2$) content (a) and level of lipid peroxidation, expressed as malondialdehyde (MDA) content (b) in *A. thaliana* exposed to mechanical stimulation (5 N, 20 s). Results are mean values of six biological replicates ± standard error. Different letters above the error bars represent significant differences ($p \leq 0.05$), based on Dunn's test

Note: C–control (untreated) plants; 1 h–leaves 1 h after mechanical stimulation; 20 h–leaves 20 h after mechanical stimulation; SR 20 h–unstimulated leaves (from treated plants) 20 h after mechanical stimulation.
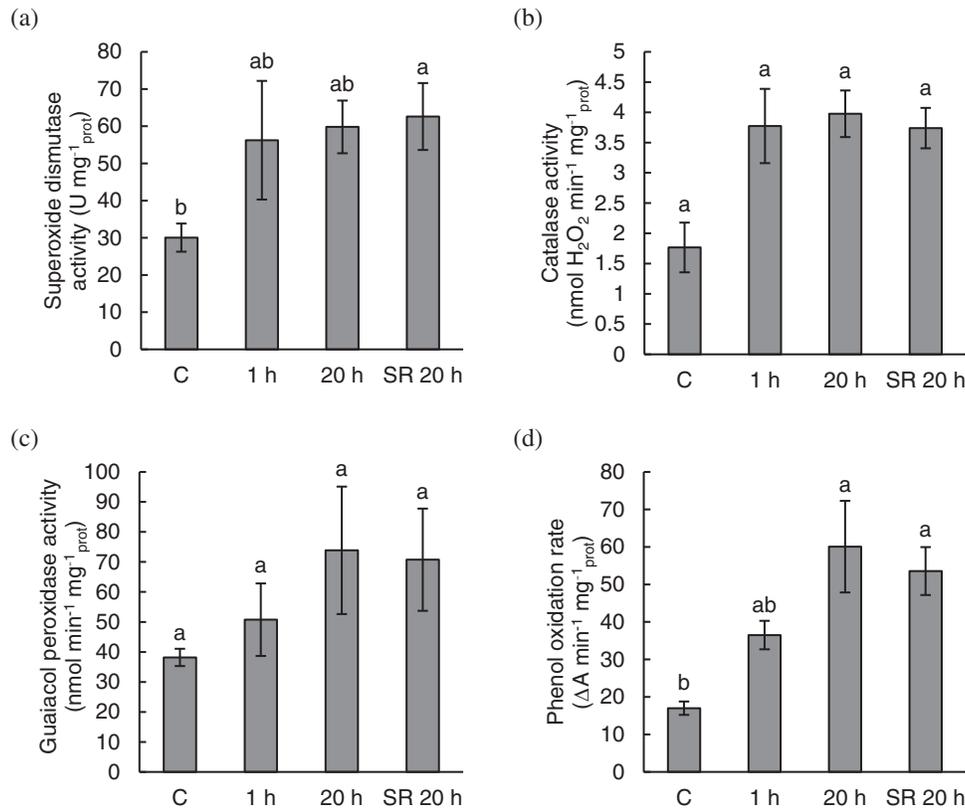


**Figure 3:** The activity of superoxide dismutase (SOD) (a); catalase (CAT) (b); guaiacol peroxidase (G-POD) (c); and phenol oxidation rate (POR) (d) in *A. thaliana* exposed to mechanical stimulation (5 N, 20 s). Results are mean values of five to six biological replicates ± standard error. Different letters above the error bars represent significant differences ($p \leq 0.05$), based on Dunn's test

Note: C–control (untreated) plants; 1 h–leaves 1 h after mechanical stimulation; 20 h–leaves 20 h after mechanical stimulation; SR 20 h–unstimulated leaves (from treated plants) 20 h after mechanical stimulation.

Compared with control leaves, unstimulated leaves analysed for systemic response (SR 20 h) showed a significant increase in the activity of SOD. Similarly, the activity of POR in leaves analysed 20 h after stimulation was significantly increased in both groups of samples–stimulated leaves (20 h) and unstimulated leaves analysed for systemic response (SR 20 h), compared with the control leaves (Figs. 3a–3d).

### 3.3 Phenolic Content

There was no effect of mechanical stimulation on phenolic content. Only slightly lower phenolic content was observed in both stimulated and unstimulated leaves of treated plants in tissue collected 20 h after mechanical stimulation (SR 20 h). The observed differences were not statistically significant (Fig. 4).
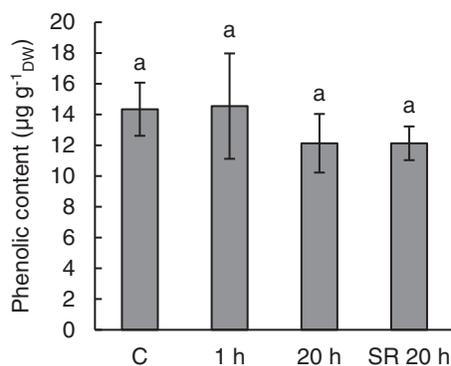


**Figure 4:** Phenolic content in leaves of *A. thaliana* exposed to mechanical stimulation (5 N, 20 s). Results are mean values of five to six biological replicates ± standard error. The same letters above the error bars indicate no significant differences between treatment groups ($p \leq 0.05$), based on Dunn's test
Note: C–control (untreated) plants; 1 h–leaves 1 h after mechanical stimulation; 20 h–leaves 20 h after mechanical stimulation; SR 20 h–unstimulated leaves (from treated plants) 20 h after mechanical stimulation.

### 3.4 Chlorophyll Fluorescence and Photosynthetic Pigment Content

In general, there was no prominent influence of mechanical stimulation on most chlorophyll fluorescence parameters and pigment content. The calculated parameters of chlorophyll fluorescence–$F_v/F_m$ and qP–were not significantly different between leaves subjected to mechanical stimulation and those of control plants (Figs. 5a and 5c). However, qP was significantly lower in unstimulated leaves sampled from treated plants 20 h after exposure to the stimuli. $\Phi_{PSII}$ was significantly lower in stimulated leaves sampled for measurement 20 h after mechanical stimulation (Fig. 5b). NPQ was significantly lower 1 h after the treatment (Fig. 5d) compared with all other groups of samples.

Although there were some perturbations in photosynthetic performance indicated by the parameters $\Phi_{PSII}$, qP, and NPQ, mechanical stimulation did not significantly alter Chl *a*, Chl *b*, and total Cars in leaves (Fig. 6). In addition, the Chl *a*/Chl *b* ratio, which is a common marker of stress, did not differ significantly between stimulated and unstimulated leaves. The calculated values ranged from 1.82 to 2.30.
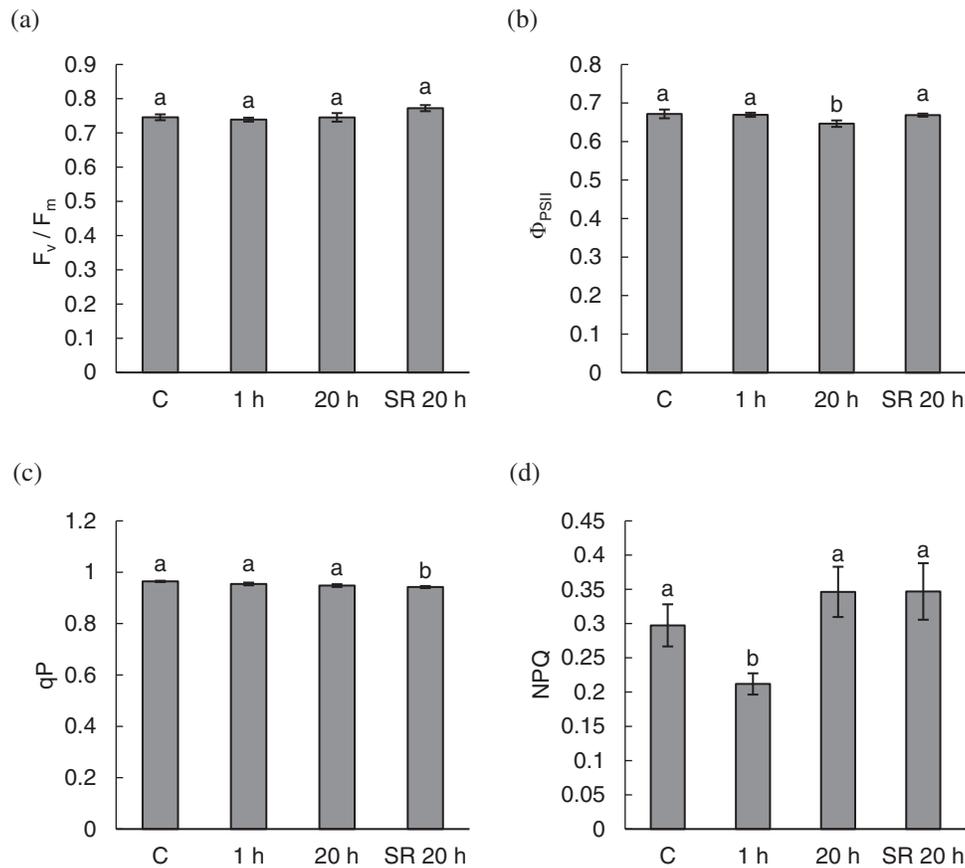
(a)

(b)

(c)

(d)



**Figure 5:** Chlorophyll fluorescence parameters–maximum quantum yield of photosystem II ($F_v/F_m$) (a); effective quantum yield of photosystem II ($\Phi_{PSII}$) (b); photochemical quenching (qP) (c) and non-photochemical quenching (NPQ) (d) in leaves of *A. thaliana* exposed to mechanical stimulation (5 N, 20 s). Results are mean values of six biological replicates ± standard error. Different letters above the error bars represent significant differences ($p \leq 0.05$), based on Dunn's test

Note: C–control (untreated) plants; 1 h–leaves 1 h after mechanical stimulation; 20 h–leaves 20 h after mechanical stimulation; SR 20 h–unstimulated leaves (from treated plants) 20 h after mechanical stimulation.

### 3.5 PCA Analysis

The main purpose of PCA analysis was to analyse the time dependence of plant response to mechanical stimulation and the difference between the response of stimulated leaves and the systemic response of unstimulated leaves.

The first step was to determine how many principal components to evaluate. The first two PCA dimensions (PCA1 and PCA2) explained 72.73% of the total variance. PCA3 explained another 10.97% of the variance, but was excluded from further analysis because it did not provide additional information. The discrimination performed with PCA analysis (Fig. 7) showed the differences in plant response to mechanical stimulation as a function of time elapsed after stimulus application. Principal component PCA1 revealed two groups–control (untreated) plants and plants analysed 1 h after the application of the mechanical stimuli, which were characterised by strong negative loadings of Chl *a*/Chl *b* ratio, Cars and MDA content, whereas plants analysed 20 h after the stimuli were characterised by strong positive loadings of SOD, G-POD, and POR activity. In addition, the principal component PCA2 distinguished control plants from plants analysed 1 h after the application of the mechanical stimuli based on the moderate positive loadings of photosynthetic parameters ($F_v/F_m$, qP) and the strong negative loadings of MDA and Chl *a* content.
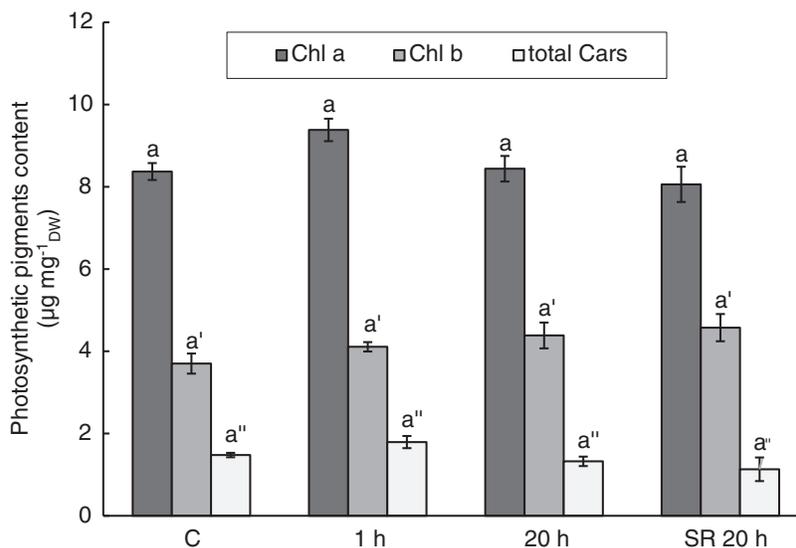
**Figure 6:** Chlorophyll *a*, chlorophyll *b*, and total carotenoids in *A. thaliana* leaves subjected to mechanical stimulation (5 N, 20 s). Results are mean values of six biological replicates ± standard error. The same letters above the error bars indicate no significant differences in chlorophyll *a* (Chl a), chlorophyll *b* (Chl b) or total carotenoids (total Cars) content between treatment groups ($p \leq 0.05$), based on Dunn's test

Note: C–control (untreated) plants; 1 h–leaves 1 h after mechanical stimulation; 20 h–leaves 20 h after mechanical stimulation; SR 20 h–unstimulated leaves (from treated plants) 20 h after mechanical stimulation.

## 4  Discussion

In this study, a moderate mechanical force of 5 N was applied to *A. thaliana* leaf blades for a duration of 20 s. The strength and duration of the stimuli did not cause immediate or permanent damage of the treated leaves or premature tissue senescence.

The application of mechanical force is expected to cause certain changes in the treated tissue, such as the disruption of the interactions between the cell wall, plasma membrane, and cytoskeleton, resulting in the transfer of information to the interior of the cell [7,19]. Like other stress factors, mechanical stimulation also leads to production of ROS [7,39]. Due to their dual function, ROS can oxidise cellular components and disrupt their function, but they also serve as a signal to activate protective mechanisms, including cell wall strengthening [38,83]. In our study, $H_2O_2$ content was similar in the leaves of control (untreated) plants and leaves sampled 1 h after mechanical stimulation, but there was a significant difference in the amount of $H_2O_2$ between leaves sampled 1 and 20 h after mechanical stimulation. According to the literature, increased levels of ROS are expected shortly after stimulation as a result of the oxidative burst that occurs immediately after stress exposure [39]. Furthermore, Benikhlef et al. [22] investigated the induction of resistance to the fungus *Botrytis cinerea* by treating *A. thaliana* with gentle mechanical stimulation in the form of soft sweeping of leaf surfaces. Immediately after stimulation, a rapid burst of ROS was detected in leaf tissue. In addition, Depège et al. [84] noted increased $H_2O_2$ content in tomato internodes a few minutes after mechanical stimulation, but this decreased slowly over the following hours. In our experiment, stimulated leaves showed significantly increased $H_2O_2$ content 20 h after mechanical stimulation compared with the value 1 h after stimulation. This observation could be explained by the involvement of $H_2O_2$ in mechanisms such as signal transduction and cell wall repair through cross-linking of protein components and lignification [84]. Moreover, it is also consistent with previously published data on biphasic ROS production, where a primary phase is expected within minutes following the exposure to stress and a secondary phase within hours or days [39]. In our work,

increased $H_2O_2$ content observed 20 h after the treatment might be due to its signalling function, as it is known that $H_2O_2$ has remarkable stability and is involved in signal transduction [85,86]. In addition, the increased rate of direct formation of $H_2O_2$ under stress conditions in various biochemical reactions and its relative stability in biological systems compared with its common precursor, the superoxide radical [87], may also contribute to its increased level 20 h after the treatment.
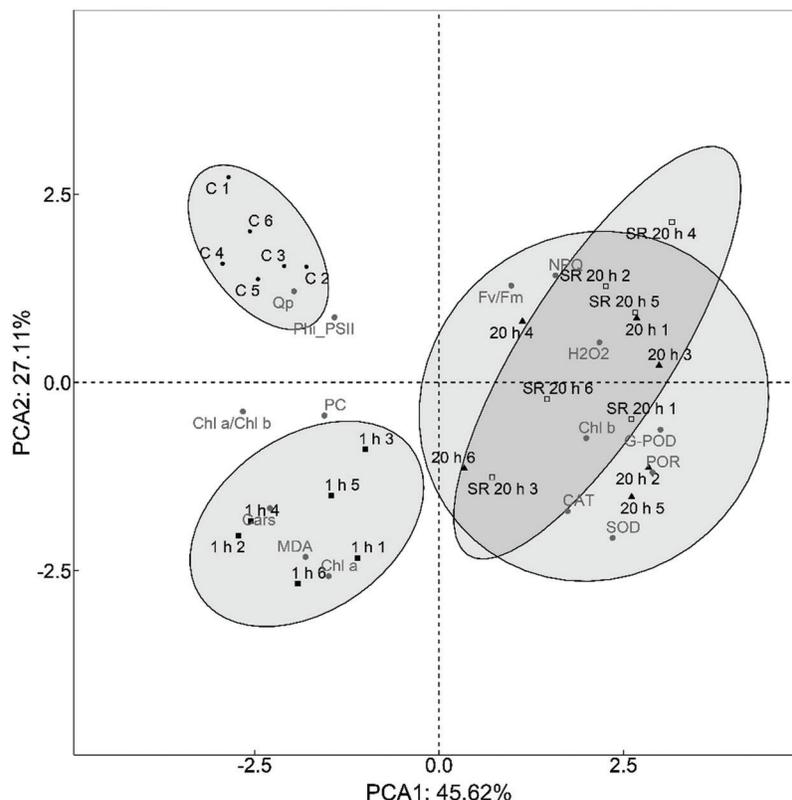


**Figure 7:** Principal component analysis biplot showing relationship between 15 measured parameters and treatment groups (C, 1 h, 20 h and SR 20 h)
Abbreviations for treatment groups: C–control (untreated) plants; 1 h–leaves 1 h after mechanical stimulation; 20 h–leaves 20 h after mechanical stimulation; SR 20 h–unstimulated leaves (from treated plants) 20 h after mechanical stimulation. Numbers 1–6 represent biological replicates.
Abbreviations for measured variables: CAT–catalase activity, SOD–superoxide dismutase activity, G-POD–guaiacol peroxidase activity, PC–phenolic content, POR–phenol oxidation rate, $H_2O_2$–hydrogen peroxide content, MDA–level of lipid peroxidation, Chl a–chlorophyll $a$ content, Chl b–chlorophyll $b$ content, Cars–carotenoids content, Chl a/Chl b–ratio chlorophyll $a$/chlorophyll $b$, qP–photochemical quenching, NPQ–non-photochemical quenching, $F_v/F_m$–maximum quantum yield of PSII, Phi_PSII–quantum yield of PSII.

Increased MDA content indicates lipid peroxidation of cell membranes caused by environmental stress in plants [45]. In our experiment, a remarkable increase in lipid peroxidation level was confirmed in leaves sampled 1 h after mechanical stimulation. The oxidative damage of membrane lipids could be induced by ROS that accumulates after abiotic stress, including mechanical stimulation [7]. Since $H_2O_2$ may be involved in the production of MDA as one of the elements of ROS [41,88,89], increased $H_2O_2$ content could be expected in leaves with increased MDA, that is 1 h after stimulation. On the contrary, the amount of $H_2O_2$ was similar to that in control plants, as mentioned previously. A possible explanation for this observation is the increased activity of CAT, an antioxidant enzyme that scavenges $H_2O_2$.

Considering the MDA level 20 h after stimulation, it was found that it returned to the control level. This could be explained by the trend of increased activity of antioxidant enzymes, suggesting that cells had sufficient capacity to limit the production of ROS and prevent further oxidative damage. Data on the increased level of lipid peroxidation induced by mechanical stress have been described in studies by other authors. Prasad et al. [29] detected products of lipid peroxidation in a short time after mechanical injury in leaves of *A. thaliana* using confocal laser scanning microscopy. Reis et al. [17] demonstrated the increased level of lipid peroxidation in *Theobroma cacao* plants subjected to mechanical stress caused by wind.

Compared to leaves of control plants, unstimulated leaves of plants subjected to mechanical stimulation showed a significant increase in SOD activity 20 h after the treatment. The activities of other two antioxidant enzymes, CAT and G-POD, slightly increased in both stimulated and unstimulated leaves of plants exposed to stress. Increased peroxidase activity after mechanical stimulation is well known and has been described by other authors. For example, Saidi et al. [68,69] described the induction of soluble peroxidases in internodes of young tomato plants 24 h after gentle rubbing. The reduced elongation of rubbed internodes observed in these studies was explained by a stiffening of the cell wall in which peroxidases were involved. A number of studies indicated the induction of peroxidases by different stress conditions, such as wounding and limited water availability, and their involvement in cell wall modifications including the synthesis and deposition of phenolics and the cross-linking of cell wall components using $H_2O_2$ as a co-substrate [90].

Activation of peroxidases and some enzymes of the phenylpropanoid pathway, which is the starting point for biosynthesis of lignin precursors and other phenolic compounds, has been reported as one of the most universal biochemical responses to environmental stress, including mechanical stimulation [19,91]. Since phenolic compounds play multiple and diverse roles in plant stress responses, i.e., structural components of the cell wall, regulation of growth and development, defence responses to abiotic and biotic stresses [55], we considered them as a possible biochemical marker of applied mechanical stimulation, and therefore, total phenolic content was also measured in this study. However, in this work, no changes in the amount of total phenolics were detected in the treated plants compared to the control and no differences were detected between the treatment groups.

Since PPOs, which are nearly ubiquitous enzymes in plants involved in many metabolic reactions including response to stress, have not been identified in the genus *Arabidopsis* [57,92], it appears that their role is compensated by laccases and peroxidases [58]. These two enzymes oxidise phenolic compounds [93], and as such may play a role in cell wall stiffening. We recorded a significant increase of POR in both stimulated and unstimulated leaves of treated plants 20 h after mechanical treatment. Compared to the control group, these samples had more than three times higher level of POR. This suggests that phenolics oxidation may play an important role in the systemic response of plants. Therefore, POR might be involved in the cell wall changes induced by mechanical stress. Interestingly, although a change of POR was observed in both stimulated and unstimulated leaves of treated plants 20 h after treatment, no difference in phenolic content was observed. These results could be explained by the increased accumulation of reducing interfering compounds during and after mechanical stimulation, such as ascorbic acid and reducing sugars, which in turn inaccurately affect the estimation of total phenolics [94]. Indeed, the study by Šic Žlabur et al. [13] showed that mechanical stimulation in the form of brushing induced a significant accumulation of ascorbic acid in leaves of lettuce and green chicory. Since the content of specific phenolic compounds can vary under different environmental conditions [53], data on the role of specific phenolic compounds are an interesting topic for future research on the response of plants to mechanical stimuli.

To assess the effects of mechanical stimulation on plant primary metabolism, photosynthetic performance of stimulated leaves of *A. thaliana* was evaluated using chlorophyll fluorescence analysis. Using several parameters, this method provides information on the status of PSII, which is indicative of

the overall rate of photosynthesis [81]. The parameter $F_v/F_m$ is a measure of the maximum efficiency of PSII. According to Lichtenthaler et al. [95], the normal value of this ratio is 0.74–0.85. In our experiment, the values ranged from 0.74 to 0.77, with no significant difference between control and stimulated leaves. $\Phi_{PSII}$ refers to the efficiency of PSII achieved and indicates the proportion of light absorbed by chlorophyll associated with PSII used in photochemistry [81]. $\Phi_{PSII}$ was decreased in stimulated leaves 20 h after the treatment, which may indicate some proportion of inactive PSII reaction centres. Elevated $H_2O_2$ content measured in these leaves at the same time point could possibly have resulted in damage to the components of the photosynthetic apparatus and lowered PSII efficiency. Photochemical quenching, qP, indicates the fraction of PSII reaction centres that are open, i.e., able to accept electrons, and a change in qP is due to the closure of reaction centres as a result of the saturation of photochemical reactions by light [81]. In our experiment, qP was decreased in unstimulated leaves 20 h after treatment. Although fluctuations in chlorophyll fluorescence often indicate changes in photosynthetic activity, Baker et al. [62] suggested that many metabolic reactions in leaves that are not directly involved in the photosynthetic process can affect the rate of synthesis of intermediates for photosynthetic reactions and thus indirectly affect photosynthesis and chlorophyll fluorescence parameters. Such perturbations of metabolism could be expected during the induction of a response to stress, including mechanical stimulation. Another quenching parameter, NPQ, involves photoprotective processes that remove excess excitation energy from chlorophyll-containing complexes and prevent the formation of harmful free radicals [96]. An increase in NPQ, as a measure of the efficiency of heat dissipation, occurs either as a result of processes that protect the leaf from light-induced damage or as a result of the damage itself [81]. Because the plants in our experiment were not exposed to high light intensity, the value of NPQ was generally low, but compared with control plants, leaves exposed to mechanical stimulation showed reduced NPQ 1 h after the treatment. This is consistent with the higher lipid peroxidation level measured in the leaves at this time point, which could be a possible reason for the disrupted NPQ mechanism.

Oxidative stress in leaf tissue, detected 1 h after the treatment as increased lipid peroxidation and 20 h after the treatment as increased $H_2O_2$ content, did not result in the degradation of photosynthetic pigments. The levels of Chl *a*, Chl *b*, and total Cars were not affected by gentle mechanical stimuli. Even the Chl *a*/Chl *b* ratio, commonly used as an indicator of stress [63], was not altered. Cars are pigments that, in addition to their role in photosynthesis, could contribute to oxidative stress defence mechanisms [38]. In addition to tocopherol, they could contribute to membrane protection against ROS [97], which could cause lipid peroxidation. Therefore, the induction of higher levels of Cars was expected to be due to increased lipid peroxidation and $H_2O_2$ content. However, the lack of a significant change in Cars content may indicate that protection by Cars was not a part of the response to oxidative stress caused by mild mechanical stimulation. Our results are in agreement with a study by Šic Žlabur et al. [13], in which mechanical stimulation by brushing had no significant effect on the content of pigments, including Chl *a*, Chl *b*, and Cars.

Principal component analysis (PCA) was performed to determine if there was a time dependence of plant response to mechanical stress and to reveal the difference between the response of stimulated leaves and the systemic response of unstimulated leaves of plants exposed to mechanical stress. PCA analysis revealed three groups–the first consisted of leaves from unstimulated plants (control), the second consisted of leaves sampled 1 h after the treatment, while the third group included both stimulated and unstimulated leaves from plants exposed to the mechanical force and sampled and analysed 20 h after the treatment. The results obtained indicate a time dependence of plant response to the applied treatment. In addition, the observation that stimulated and unstimulated leaves of treated plants sampled 20 h after mechanical stimulation were grouped together may indicate signal transduction from stimulated to unstimulated leaves, that is, a systemic response in distant leaves. These results are consistent with current knowledge of local and systemic responses to other types of abiotic stresses that have been studied to date. For example, Miller et al. [83] reported a rapid systemic signal associated with the accumulation of ROS in

the extracellular space that spread at a rate of 8.4 centimetres per minute in *A. thaliana* exposed to various abiotic stress factors. In the extensive review, Baxter et al. [39] reported the contribution of $H_2O_2$ in cell-to-cell signal propagation, that is, its accumulation in extracellular spaces and generation of a "ROS wave" in systemic signalling. They also reported studies of responses to three types of abiotic stress (high light, heat, and cold) that elicited similar responses in local and systemic tissues.

## 5 Conclusion

Our study suggests that moderate mechanical stimulation is perceived by plants but does not induce prominent changes in plant metabolism and photosynthetic performance. The applied treatment resulted in a transient increase in the amount of $H_2O_2$ and level of lipid peroxidation, as well as an increase in SOD and POR. The activity of PSII was slightly affected, but there were no noticeable effects on photosynthetic pigments. PCA analysis separated three groups–1) leaves of control (untreated) plants, 2) stimulated leaves sampled 1 h after the treatment, and 3) stimulated and unstimulated leaves of treated plants 20 h after the treatment, which may indicate signal transduction from stimulated to unstimulated leaves, that is, a systemic response to a local application of mechanical stimuli in distant leaves. These results could be of particular interest in the context of applying moderate mechanical stimuli to prime and improve plant growth and yield under other adverse environmental conditions. However, more detailed physiological, biochemical, and molecular analyses are needed to identify and characterise pathways that may be part of the local and systemic plant response to mechanical stimuli, especially in agronomically important plant species.

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