



OPEN Short-term airborne ultrasound induced cell death in tobacco cells and changed their wall components

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The effects of low-intensity ultrasound on plants such as piezoelectric and ultrasonic water baths, on plants have been extensively studied. However, the specific effect of airborne ultrasound on plant cells has yet to be reported. The present study was conducted to elucidate the physiological responses of plant cells to airborne US. Homogeneous suspension-cultured tobacco cells (*Nicotiana tabacum* L. cv Burley 21) were subjected to airborne US at 24 kHz in two pulsatile and continuous modes for 10 and 20 s. The study's outcome revealed that airborne US triggered the production of H₂O₂, elevated internal calcium concentration, and reduced antioxidant capacity upon cavitation. Alteration of covalently bound peroxidase and other wall-modifying enzyme activities was accompanied by reduced cellulose, pectin, and hemicellulose B but increased lignin and hemicellulose A. The biomass and viability of tobacco cells were also significantly decreased by airborne US, which ultimately resulting in PCD and secondary necrosis. The results highlight the potential risks of even short-time exposure to the airborne US on plant physiology and cell wall chemical composition raising significant concerns about its implications.

Keywords Airborne ultrasound, Cell wall components, Cell death, Membrane integrity, Redox status, Tobacco cells

Ultrasound (US) is a powerful tool with diverse applications in medical diagnostics, therapeutics, and industry. In medicine, it is applied for diagnostic sonography (ultrasonography), reflection technology (echo), and treatment of soft tissue ailments and injuries, bursitis, and collagen diseases¹. In industry, ultrasound is used for cleaning, welding of plastics and metals, cutting, shaping, separating, mixing, and degassing². Piezoelectric US has been used in the harnessed green synthesis of well-dispersed and non-aggregated iron oxide nanoparticles using plant extracts³. The exposure to the US disintegrates cellular ultrastructure and breaks extracellular polymers, leading to cavitation, acoustic microstreaming, and eventual alteration of the cell membrane permeability. In herbal science, ultrasound is a fascinating tool that significantly enhances the release of phytochemicals from plant sources. A potential area for further exploration and research^{4,5}. For example, the implication of ultrasound increased the yield rate constant for the ultrasound extraction of saponins from alfalfa leaves almost two times more than that of routine heat-reflux methods⁶. Ultrasound has also been widely used to reduce the energy consumption and drying time of herbs^{7,8}. The effect of the US on biological systems depends on its energy level and the type of exposed cell. The thermal and chemical effects of low-frequency ultrasound on living organisms have been widely investigated⁹. Ultrasonic baths and piezoelectric-based ones were used to increase taxans yield by suspension-cultured hazel cells, and no adverse effect on the viability of cells and the integrity of their membranes occurred^{10,11}. The exposure of soybean seeds to the airborne US increased water uptake without altering the morphology and the wettability of the seed coat⁹.

Airborne power ultrasound is a green technology with significant potential for food and environmental applications. The duration and energetic performance of hot air drying of peppermint leaves in the presence of high-intensity airborne US has been studied by Ghanbarian et al.¹². Propagation of high-intensity waves by airborne US through air and multiphase media produces permanent changes in the exposed objects and substances. The nonlinear effects produced in such media are responsible for the beneficial repercussions of US in airborne applications though its adverse effects remain essentially uninvestigated^{13–16}. Airborne US is emitted primarily or as a side effect of various devices¹⁷. Various machines act as sources of airborne US, including high-frequency cutting tools, ultrasonic cleaners, welding equipment, and some laboratory and medical instruments

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such as fans, compressors, air handling units, transformers, high-voltage power lines, and electrical discharge machining¹⁷.

Despite the fact that airborne US is more frequently found in plant environments, its effects on the physiology of plant cells and the underlying mechanism(s), still need to be investigated, compared to piezoelectric and water bath-based US. As mentioned, most of the studies on the biological effects of airborne US are confined to diagnostic medical approaches in humans, and a few studies have been devoted to the physiological responses of plant cells to it. This area of research presents an exciting opportunity for further exploration. The present study investigated the physiological responses of suspension-cultured tobacco cells to 24 kHz airborne US for 10 and 20 s short periods.

Material and method

Tobacco cell culture and ultrasound treatment conditions

All methods implemented here were performed in accordance with the corresponding national guidelines and regulations. According to the research ethics committee of Tarbiat Modares University, no certificate was needed for this research. A rapid growing cell line of tobacco (*Nicotiana tabacum* L. cv Burley 21), grown in a modified LS liquid medium, was used¹⁸ to study the effects of airborne US. Suspension cultures were established from calli (calluses) of tobacco cells that had been maintained in our laboratory for more than 200 subcultures. Seven-day-old cells (in their logarithmic growth phase (Fig. 1)) were floated as one layer on the surface of a medium containing rectangular plates (20 × 40 cm) and exposed to the airborne US at a frequency of 24 kHz with two modes of pulsatile and continuous. The airborne US was produced by a locally designed device (Fapan Co, Iran). The physical structure of the airborne US probe used in the present experiment is shown on Fig. 2. The US applicator set up consisted of an ultrasonic transducer, a booster, and a Ti horn. The ultrasonic generator drives the airborne probe with a frequency of 24 kHz and an electrical power of 100 W. This ultrasonic probe generated airborne US waves and irradiated the tobacco cells at its far field region. Receiving of waves by the sample was checked with disruption and rearrangement of a layer of sodium chloride powder (Fig. 2).

The treatment duration was 10 and 20 s, and the cells were exposed 20 cm far from the probe tip. After ultrasound exposure, the cells were allowed to grow at room temperature for 24 h and then harvested under reduced pressure. The control groups were placed in similar conditions in terms of temperature and humidity. The samples' fresh weight (FW) was measured immediately, and their dry weight (DW) was determined after drying at 60 °C for 48 h. Aliquots of the cells were frozen with liquid nitrogen and kept at -80 °C for further analysis.

Redox and membrane integrity assay

The H₂O₂ content of the control and airborne US-treated cells was determined based on the iodine level produced due to the interaction of hydrogen peroxide with iodide ions¹⁹. The ferric ion-reducing antioxidant

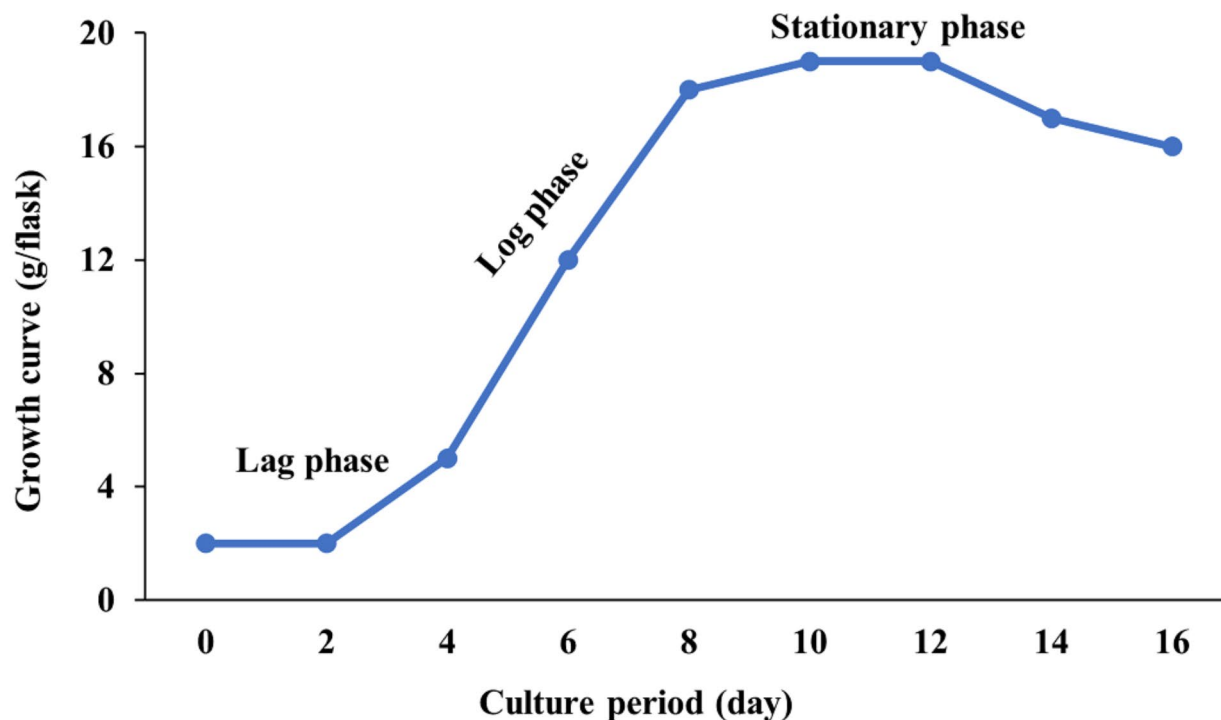


Fig. 1. Growth curve of tobacco cells in LS medium.

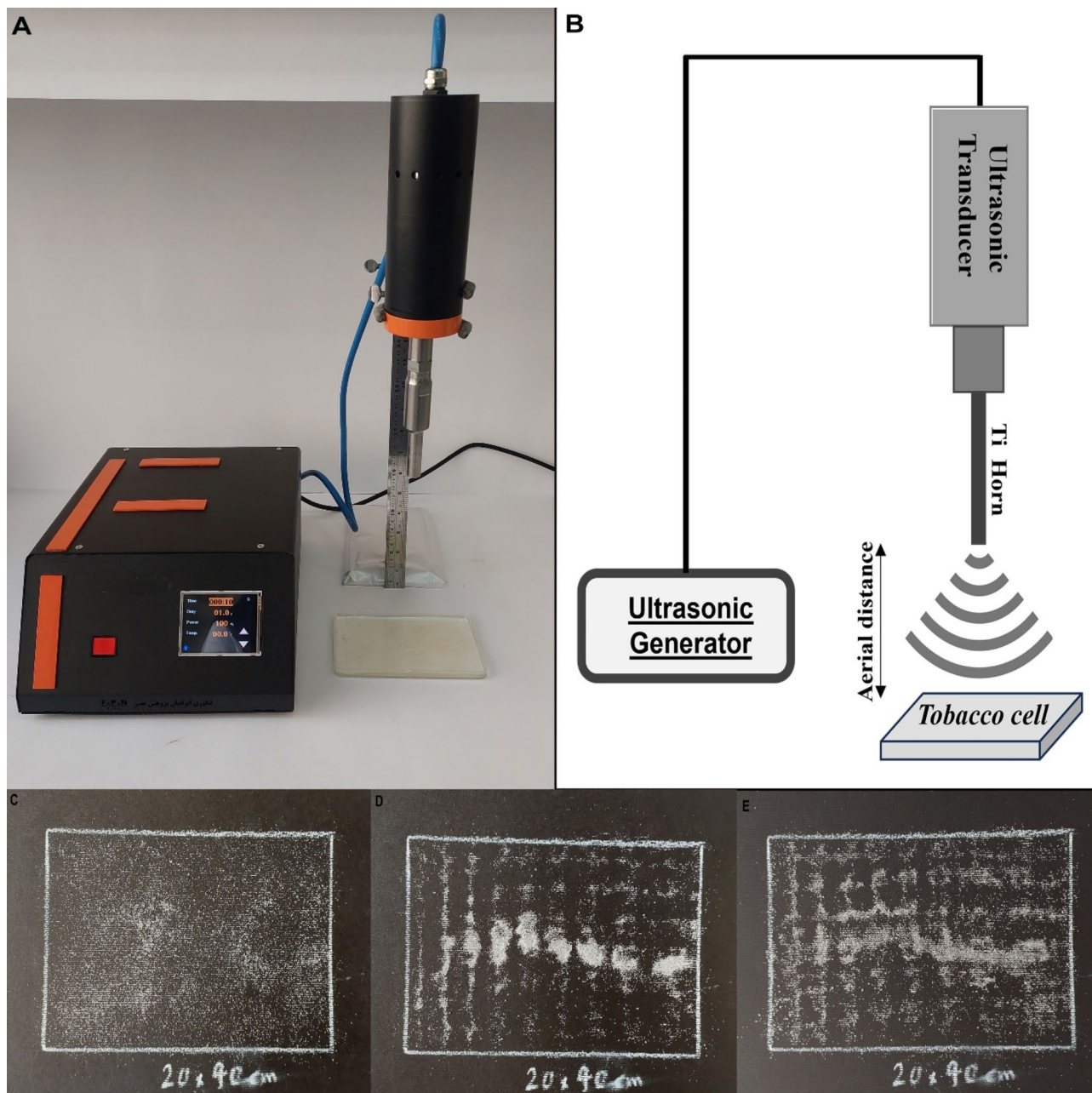


Fig. 2. Airborne US generator and procedure of irradiation. (a, b), Real and schematic shapes of the device. Tobacco cells containing plates were placed at a 20 cm distance from the tip. Receiving of waves by the sample was checked with disruption and rearrangement of a layer of sodium chloride powder, (c) before irradiation, (d, e) after irradiation with airborne US at continuous and pulsatile modes, respectively.

power (FRAP) in cells was assayed based on the oxidation-reduction of potassium ferricyanide and ferric chloride in the presence of trichloroacetic acid²⁰. Total radical scavenging capacity (RSC) was determined using the stable radical 2,2-diphenyl picrylhydrazyl²⁰.

Malondialdehyde (MDA) was considered the final product of the peroxidation of membrane lipids, and it was measured by thiobarbituric acid reactive substances. The absorbance of the supernatant was measured at 532 and 600 nm. The value for non-specific absorption at 600 nm was then subtracted from that of 532 nm¹⁹.

To clarify the effects of US on electrolyte leakage (EL) through membranes, the harvested cells were washed thoroughly and suspended in double distilled water at 25 °C for 30 min, and the electrical conductivity (E1) of the medium was measured. Subsequently, cells were treated with boiling water for 60 min, cooled at room temperature and the resulting electrical conductivity (E2) was measured. The EL was calculated from the proportion of E1 to E2²¹.

The viability of the cells was evaluated through microscopic analysis and quantified using Evans Blue by a light microscope (BH2, Olympus, Tokyo, Japan) equipped with a digital camera¹⁸.

Estimation of protein and soluble sugar contents

Proteins were determined by Coomassie brilliant blue G250 in acidic pH, and soluble sugar content was determined based on the reaction of phenol and sulfuric acid using a UV-visible double-beam spectrophotometer (GBC-Cintra6, Australia)²¹.

Cytosolic calcium concentration

The concentration of cytosolic free Ca^{2+} was determined by loading cells with the acetoxymethyl ester of the Ca^{2+} -binding dye Fura-2 (Fura-2-AM, Molecular Probes, Sigma-Aldrich, USA) in the dark at 28 °C for 1 h. The cells were then washed three times with fresh medium and left for 15 min to have the Fura-2 crossed cell membranes and entered the cells where the cytosolic esterases cleaved the acetoxymethyl hydrophobic side chains and produced the hydrophilic permeable fluorescent dye/ Ca^{2+} complex. Ca^{2+} -bound Fura-2 AM had an excitation maximum of 340 nm, while the Ca^{2+} -free Fura-2 AM maximum excitation happened at 380 nm. In both states, the maximum emission was about 510 nm²².

Cell wall components assay

The cell walls were isolated using EtOH, a mixture of CHCl_3 : MeOH (1:1 v/v), and acetone by subsequent filtration in each step and final drying. From the dried wall powder, pectin was extracted stepwise with hot ammonium oxalate (20 mM, 70 °C) and NaOH 0.1 M then freeze-dried and weighed. A solution of sodium hydroxide (17.5%) containing sodium borohydride (0.02%) was used to extract hemicellulose from the residue of the former step. By adding HOAc, hemicellulose A (HA) was precipitated, and the supernatant was freeze-dried and defined as hemicellulose B (HB). After washing, the cellulose was isolated, and its weight was determined²³.

Major wall-bound phenolic acids were liberated from pectin with EtOAc, air-dried, re-dissolved in 50% MeOH, and determined by HPLC (Waters, e2695, USA) equipped with C18 column (Perfectsil Target ODS3, 5 μm , 250 \times 4.6 mm, MZ-Analysentechnik, Mainz, Germany). Phenolics were eluted at a flow rate of 0.5 mL min^{-1} with a linear gradient of 30–80% MeOH containing 0.1% HOAc and were detected at 280 nm using a 2489 UV-vis detector²⁴.

A modified acetyl bromide procedure was used to determine lignin content. Fine powder of dried cell wall was suspended in a mixture of AcBr in HOAc (25%, w/w), followed by the addition of 100 μL of HClO_4 (70%) and subsequently heated at 70 °C for 30 min. The mixtures were shaken at 10 min intervals. After cooling, the digestion mixture was added to the NaOH (2 N) solution and scaled up with HOAc. The lignin content was measured at 280 nm using a specific absorption coefficient of 20.0 $\text{g}^{-1} \text{L cm}^{-1}$ ²³.

Peroxidase activity was measured in soluble, ionically-, and covalently- wall-bound fractions (SPO, IPO, and CPO, respectively). Guaiacol was used as a substrate of SPO, and syringaldazin was used for wall-bound IPO and CPO¹⁸.

Cellulase activity was measured based on the reduction of 3,5-dinitro salicylic acid to the corresponding 3-amino 5-nitrosalicylic acid in the presence of 3,5-dinitro salicylic acid that was manifested by a change of color from yellow to brick red²⁵. Endoglucanase (EGase) activity was determined based on the release of glucose from carboxymethyl cellulose²⁶. The glucose concentration was measured by the phenol sulfuric method and correlated to the endoglucanase.

Statistical analysis

The experiments were conducted based on a completely randomized design with three independent repetitions. To ensure the robustness of our analysis, we employed advanced statistical tools such as SPSS (version 22, USA) and ANOVA to justify the effectiveness of the treatment and significance of the changes observed. Significant differences were defined using Duncan's tests at the 0.05 confidence level. The principal component analysis (PCA) and hierarchical cluster analysis (HCA) were computed through the algorithm embedded in the web-based metabolon software, (<http://www.metaboanalyst.ca>).

Results

Redox status and calcium content of the cells

Continuous exposure to the airborne US significantly increased the concentrations of H_2O_2 and MDA in tobacco cells (Fig. 3A, B). However, when cells were exposed to the pulsatile mode of airborne US, no difference between the H_2O_2 and MDA contents of treated cells and control ones was identified.

The application of continuously airborne US on tobacco cells for 10–20 s, reduced the antioxidant and free radical scavenging capacities of the cells, compared to their corresponding controls (Fig. 3C, D). Pulsatile mode, however, only reduced the RSC of the cells and did not affect FRAP, in comparison with their controls (Fig. 3C, D). The effect of airborne US on membrane permeability was assessed by monitoring the leakage of electrolytes. Both pulsatile and continuous airborne US treatments increased the cells' EL (Fig. 3E). Furthermore, continuous exposure to airborne US increased cytosolic calcium of tobacco cells but pulsatile mode caused no significant changes in cytosolic calcium concentrations of the cells (Fig. 3F).

Effect of the US on the viability, growth, and morphology of tobacco cells

Continuous exposure of cells to the airborne US decreased the DW, protein, and sugar contents of tobacco cells (Table 1). The viability percentage showed a significant reduction after 20s continuous exposure to airborne US, however, FW decreased after 10s exposure and remained unchanged after 20s (Table 1). On the other hand, pulsatile airborne US reduced the viability and DW but didn't affect FW after both 10 and 20s exposure (Table 1). Protein and soluble sugar decreased after 20s pulsatile exposure to airborne US while, 10s exposure had no effect compared to their corresponding control (Table 1).

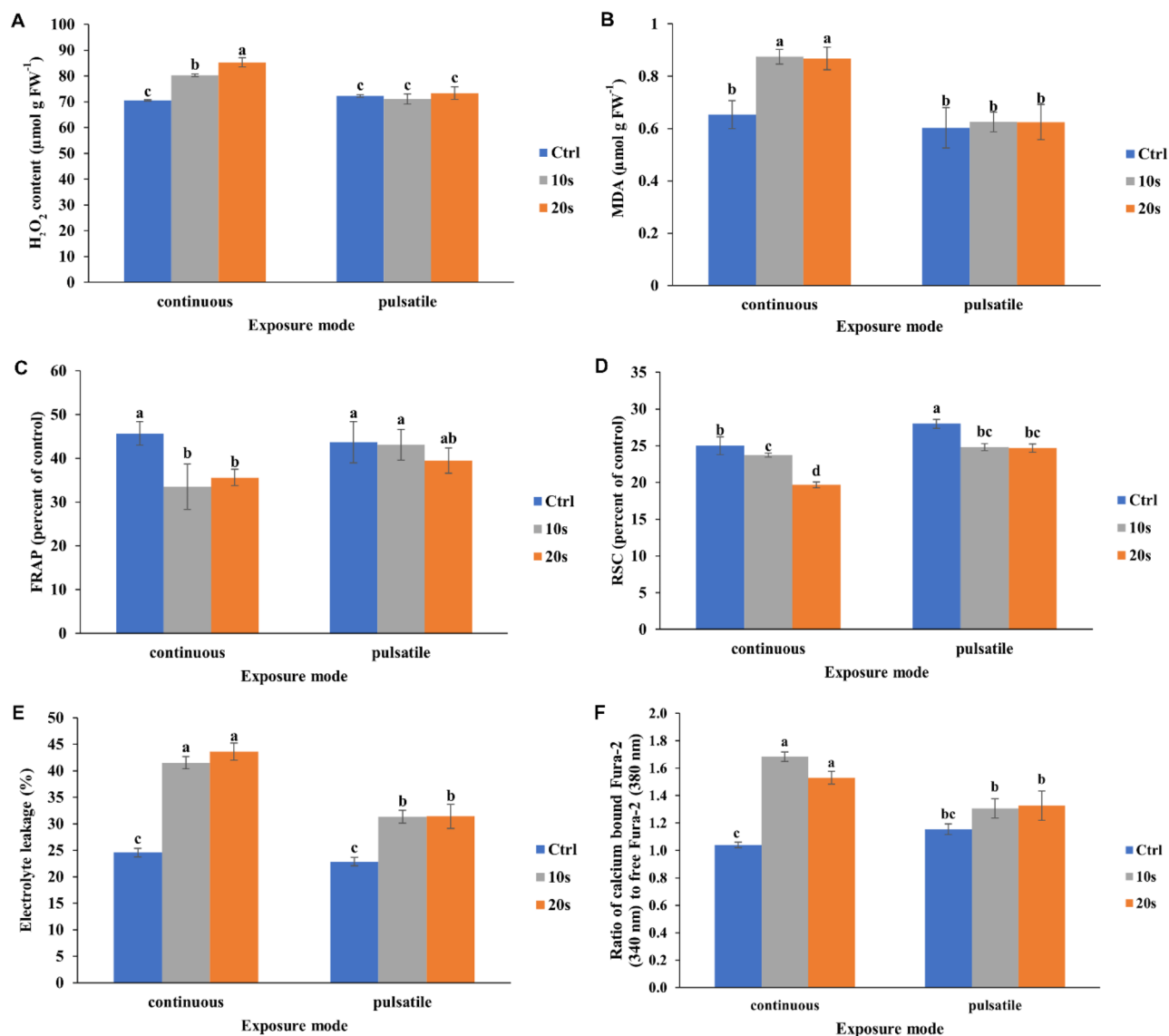


Fig. 3. Effect of airborne US of 24 kHz at continuous and pulsatile modes on redox parameters of tobacco cells. (A) H_2O_2 , (B) MDA, (C) FRAP, (D) RSC, (E) electrolyte leakage, and (F) cytosolic calcium are shown. Different letters denote significant differences according to the Duncan test ($n=3$, $p\leq 0.05$).

Treatment Modes	Exposure time (s)	Viability (%)	Fresh weight (g)	Dry weight (g)	Protein (mg g FW^{-1})	Soluble sugars ($\mu\text{g g FW}^{-1}$)
pulsatile	0	95.66 ± 1.5 ^a	12.58 ± 0.2 ^a	7.69 ± 0.2 ^a	90.85 ± 6.9 ^a	315.5 ± 28 ^a
	10	93 ± 5.6 ^b	12.22 ± 0.7 ^a	6.34 ± 0.3 ^{bc}	92.66 ± 9.5 ^a	314.2 ± 18 ^a
	20	82.66 ± 2.6 ^c	12.15 ± 0.2 ^a	6.58 ± 0.1 ^b	55.72 ± 8.7 ^c	240.3 ± 6.3 ^b
continuous	0	96 ± 1 ^a	12.26 ± 0.3 ^a	7.58 ± 0.2 ^a	96.45 ± 8.7 ^a	314.2 ± 6.9 ^a
	10	89.33 ± 5.5 ^{ab}	10.17 ± 0.6 ^b	5.81 ± 0.1 ^d	80.14 ± 3.9 ^b	252.9 ± 4.9 ^b
	20	83.33 ± 3.5 ^{bc}	11.36 ± 0.5 ^{ab}	6.07 ± 0.1 ^{cd}	52.79 ± 6 ^c	267.4 ± 7.8 ^b

Table 1. Growth characteristics of tobacco cells before and after treatment with 24 kHz airborne US. Different letters indicate significant differences according to the Duncan test ($n=3$, $p\leq 0.05$).

Figure 4 shows the results of microscopic observation of tobacco cells and the damage of their membranes before and after exposure to airborne US. Compared to the control cells and their intact membranes (Fig. 4A), continuous mode caused rupture and blabbing in cell membranes after 10 and 20s exposure which shows with arrows in Fig. 4B-D. Adverse effects of pulsatile US on membranes of exposed tobacco cells were confined to projections, but no obvious rupture was observed (Fig. 4C-F).

Effects of US on the cell wall components and modifying wall enzymes of exposed cells

Exposure to airborne US in both continuous and pulsatile modes significantly increased total cell wall chemical materials (CWM), compared to non-treated cells (Fig. 5A). Among polysaccharide components present in the cell wall, in comparison with the control group, pectin, HB, and cellulose were reduced after exposure to pulsatile airborne US at both exposure durations (Fig. 5B, C, E). In contrast, continuous US mode resulted in a significant reduction of pectin after 10s, with no notable changes observed after 20s. Cellulose remained unchanged after 10s of continuous exposure but exhibited a reduction after 20s. Additionally, HB decreased after continuous US exposure at both time intervals. The content of HA in the airborne US-treated tobacco cells was significantly higher than that of the control (Fig. 5D). The content of lignin in airborne US-treated cells at continuous mode was considerably higher than that of the control, while no significant changes were observed between lignin content of pulsatile airborne US-treated and non-treated cells (Fig. 5F).

The activity of EGase was significantly increased (Fig. 6A), indicating potential changes in enzymatic activity due to the treatment. This increase in EGase activity suggests a potential alteration in the cell wall structure. Similarly, the activity of cellulase also increased, particularly with continuous airborne US, showing a clear impact of the treatment on enzyme activity, however in the case of pulsatile mode, the enzyme activity increased only when cells were exposed to airborne US for 10 s (Fig. 6B).

The effect of airborne US on the activity of different fractions of peroxidase enzyme was also investigated. The activities of SPO and IPO were lower than control groups in all modes and treatment duration in airborne US-treated cells except for SPO that remained unchanged even after 20 s exposure to continuous US (Fig. 6C, D). The activity of CPO, however, was higher than the control only in continuous mode (Fig. 6E).

Effects of airborne US on the cell wall phenolic content

Among the detected wall-bound phenolic acids, the content of the cinnamic-, ferulic-, and P-coumaric acids significantly increased after continuous exposure to the airborne US, while no significant change was observed in caffeic acid content (Fig. 7A-D). These findings have significant implications for our understanding of the effects of airborne US on plant cell walls. Under pulsatile airborne US treatment, cinnamic- and ferulic acid content did not change, whereas that of the caffeic acid increased only after 20 s and p-coumaric acid increased after 10 and 20 s (Fig. 7A-D).

Correlations among various parameters in cells exposed to airborne US treatment

A thorough PCA was conducted to identify the correlations between various parameters providing a clear understanding of the effects of the airborne US on treated cells compared to control (Fig. 8). The loading plots from PCA demonstrated a high degree of accuracy in classifying the tested parameters, with a total variance of 84.1%. This analysis suggests clear differences between 10 and 20 s airborne US-treated and untreated cells. The PCA data graph (PC1 and PC2) of the PCA data emphasizes the differences and the variability of airborne US treatment (continuous and pulsatile) is depicted by component 1. The response to continuous airborne US treatment was particularly pronounced, showing a significantly high percentage and revealed a larger effect on the cells.

Moreover, all evaluated parameters were shown based on a similarity between the clusters pattern, which was performed by the Pearson correlation coefficient (Fig. 9). As a result, there are positive correlation patterns identified among MDA, H_2O_2 , EL, and various wall stiffening compounds in airborne US-treated cells (Fig. 9). On the other hand, the results of this study confirmed negative correlation between viability, FW, and DW and the parameters mentioned above of tobacco cells, indicating a potential decrease in the parameters with airborne US treatment.

Discussion

It is widely acknowledged that US induces acoustic microstreaming and cavitation in liquid media²⁷. This phenomenon, known as sonolysis. Sonolysis of water molecules and thermal dissociation of oxygen molecules upon cavitation is plausible in both the external liquid LS medium and in the cytosol of tobacco cells, as given that almost 75% of their body is composed of water²⁷. Most biological effects of ultrasonic irradiation are primarily attributed to the production of hydroperoxyl and hydroxyl radicals and H_2O_2 during the exposure period²⁶⁻²⁸. Compared with OH and OOH radicals, hydrogen peroxide, which has a longer lifetime, is the most reliable species to monitor the redox status of the cells²⁷. The significant increase of H_2O_2 in continuously airborne US-treated tobacco cells along with the time can be attributed to the sustained creation, growth, and oscillations of gas bubbles inside them²⁹. This phenomenon damaged the membranes of the cells and resulted in a 34% and 78% increase in MDA and EL, respectively. Damage to the plasma membrane was obviously evident in microscopic observations of airborne US-treated cells. Also, 26% reduction in FRAP and 21% decrement in RSC were identified in these cells, which demonstrates the adverse effects of airborne US on the antioxidant capacity of treated tobacco cells.

Continuous exposure of tobacco cells to the airborne US increased their internal calcium concentration by up to 160%. It has been widely accepted that calcium functions as a major secondary messenger molecule in plants under various stress conditions^{30,31}. The cavitation and acoustic microstreaming generated by the airborne US can also induce an intercellular Ca^{2+} increase³². Both increased cytosolic Ca^{2+} and H_2O_2 can induce an active

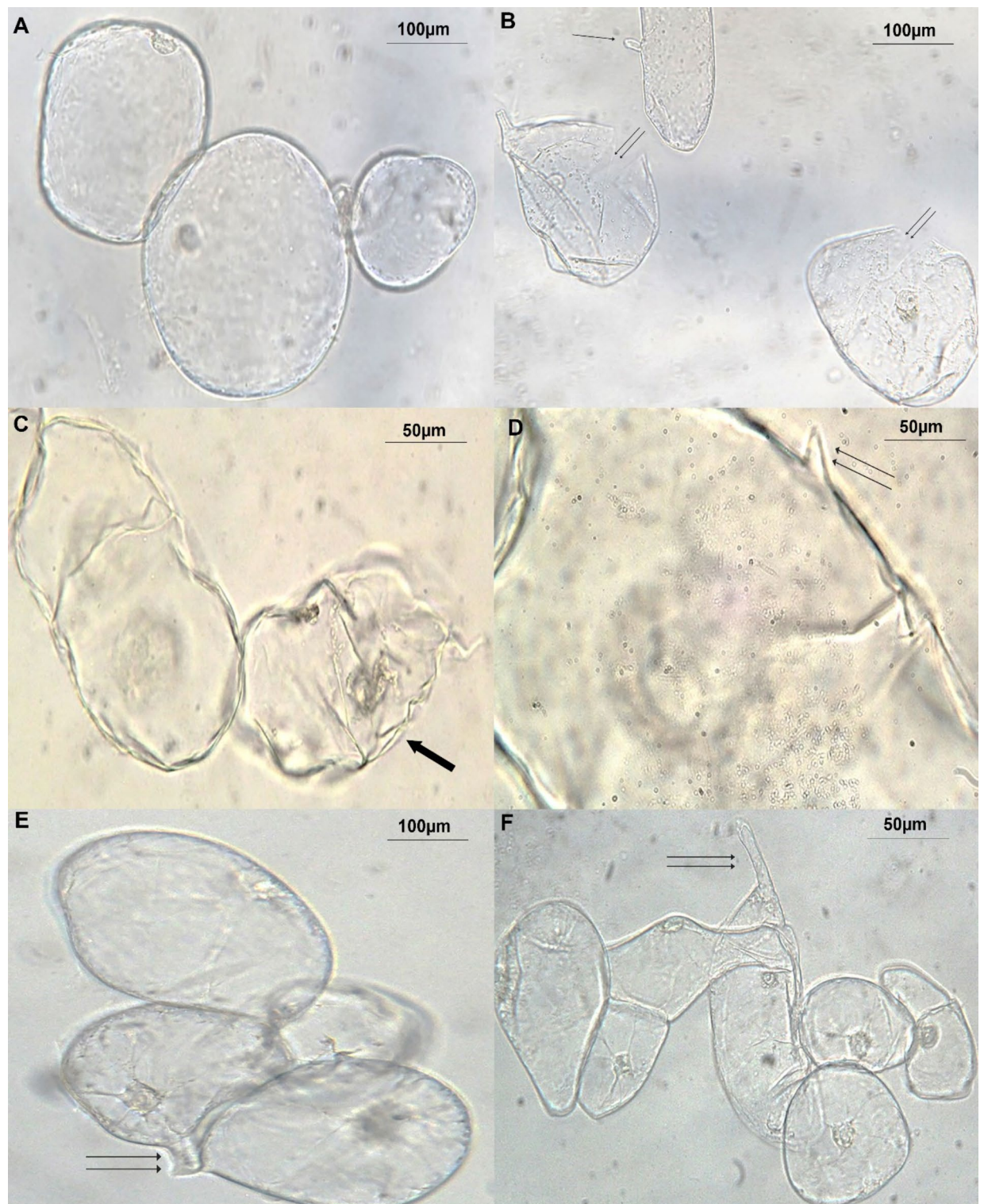


Fig. 4. Morphological features of tobacco cells before and after treatment with the airborne US, visualized under light microscope. (A) Control tobacco cells, (B–C) Continuously airborne US-treated tobacco cells after 10 and 20s, respectively. (D) Observation of membrane blabbing and projections in 20s continuously treated cells with higher magnification. (E,F) pulsatile airborne US-treated tobacco cells after 10 and 20s, respectively. The typical alterations were observed almost in 15% of cells under two modes of airborne US treatments.

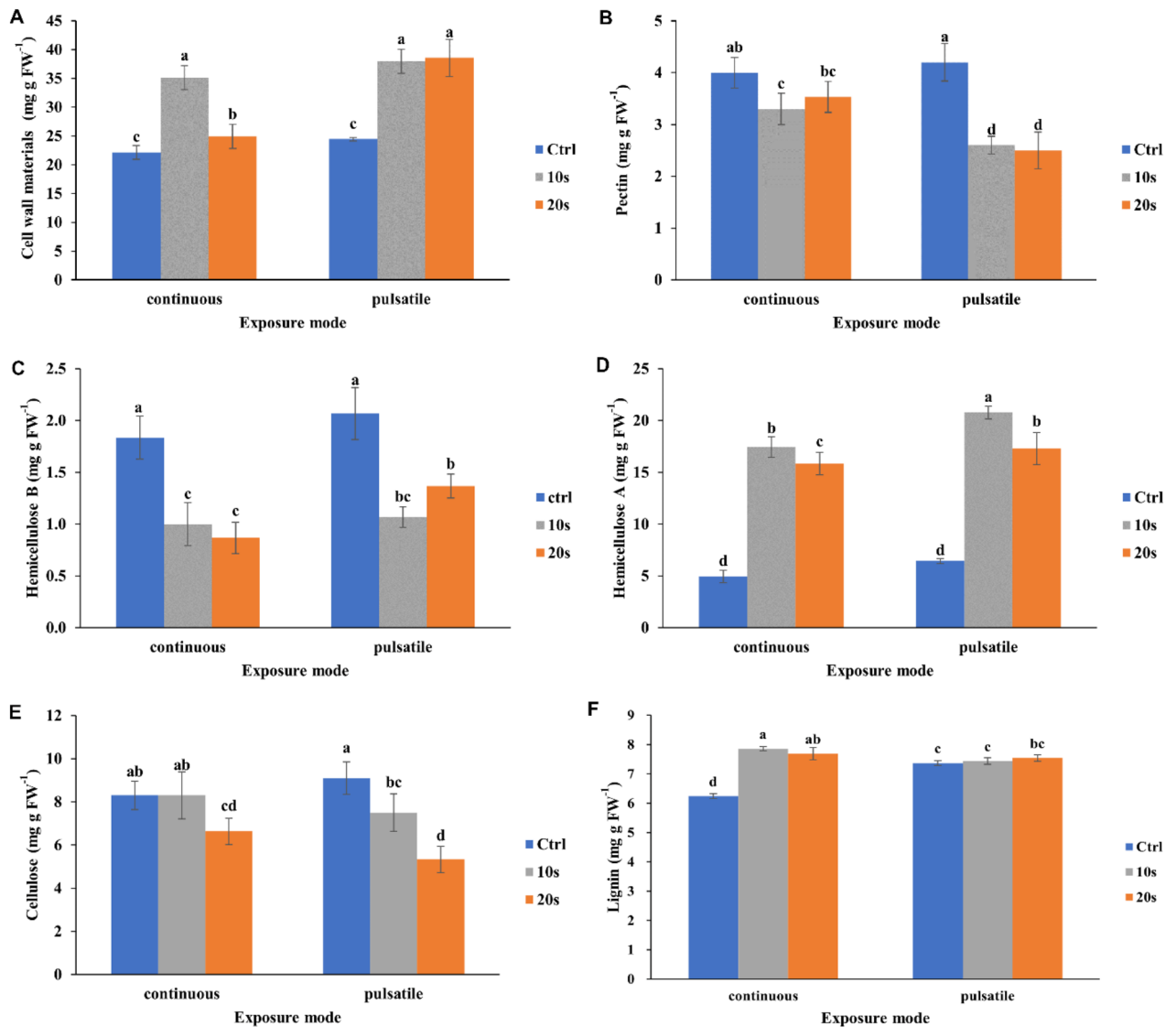


Fig. 5. Airborne US affect the quantity of tobacco. (A) cell wall components, (B) Pectin, (C) hemicellulose B, (D) hemicellulose A, (E) cellulose, (F) lignin of tobacco cells before and after treatment with 24 kHz ultrasound fixed frequency in tobacco cells are shown. Different letters indicate significant differences according to the Duncan test ($n = 3$, $p \leq 0.05$).

cell death process³³. These implications of our research on the role of calcium and H_2O_2 in inducing cell death under ultrasonic exposure conditions are of significant interest to the scientific community, engaging them in further exploration of this area. Various cell death modes triggered by airborne ultrasonic wave exposure provide a deeper understanding of plant cell growth and viability. Cell viability may be concomitantly affected by triggering various cell death modes, including primary necrosis (instant cell lysis), programmed cell death, and secondary necrosis. The latter two modes have been observed in tobacco cells because of the slow time bio-effects of the US elicited many hours after exposure³⁴. Similarly, in the present study, certain characteristics of programmed cell death (apoptosis) including the destruction of the plasma membrane and the emergence of some protrusion were observed in tobacco cells 24 h after exposure to continuous airborne US. Some hallmarks of secondary necrosis such as cytoplasmic destruction and cell damage were also observed in certain continuous airborne US-treated cells.

In comparison, exposure to the pulsatile airborne US did not bring significant changes in H_2O_2 , MDA, or internal Ca^{2+} concentration. However, the ferric oxide reduction power of the cells increased EL and reduced RSC to 137% and 88% of the control level. The increase of EL identified in both airborne US modes treated cell groups clearly denotes the US potential in the generation of acoustic cavitation and puncturing of the cells, sparking further interest in the potential applications of ultrasonic technology³⁴.

Under both modes of airborne US exposure, the antioxidant capacity of cells was significantly reduced, leading to an oxidative burst and a subsequent decrease in the viability and dry and weight of tobacco cells. This

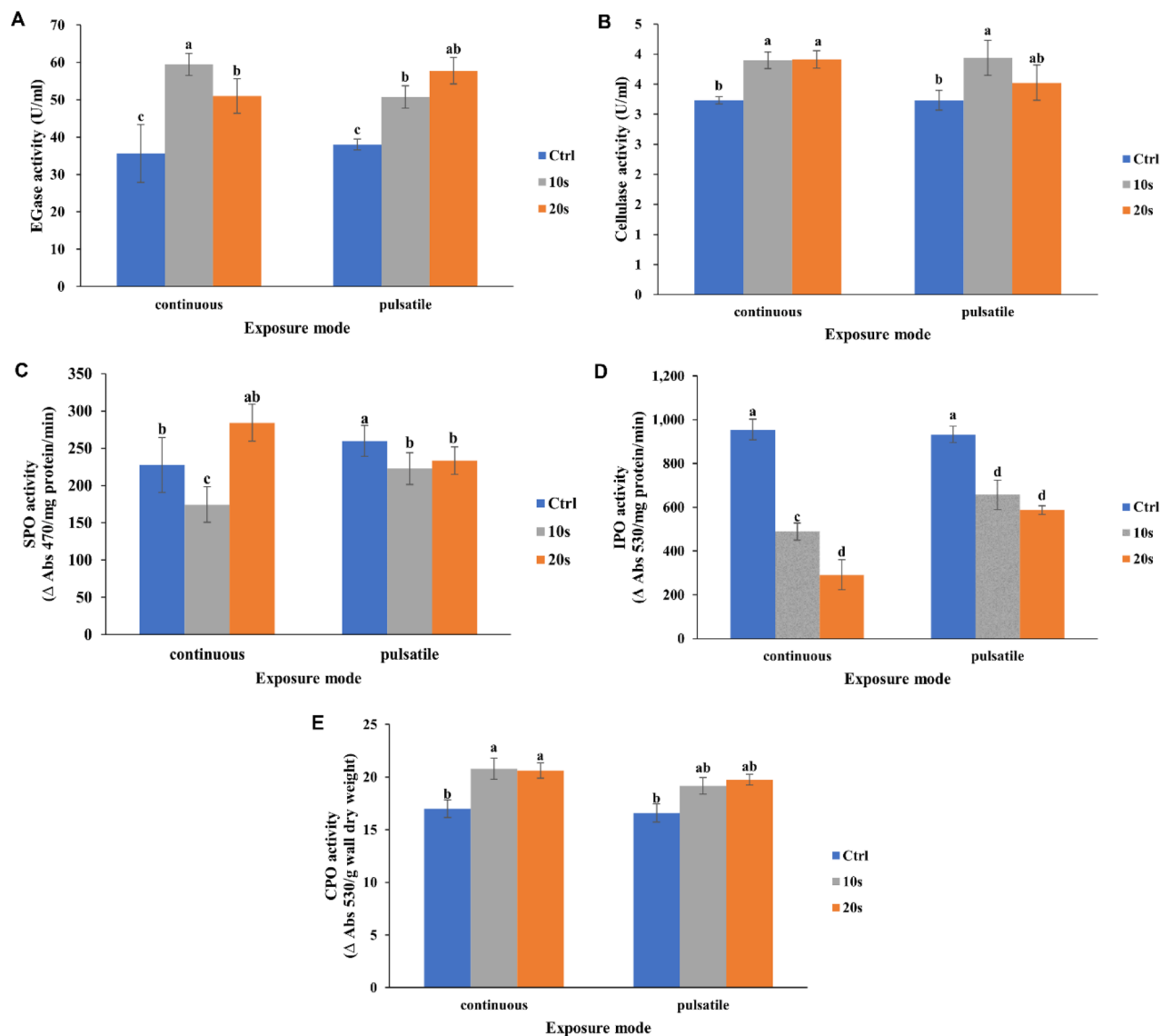


Fig. 6. Effect of airborne US on the activity of wall modifying enzymes. The activity of (A) EGase, (B) Cellulase, (C) SPO, (D) IPO, and (E) CPO are shown. Different letters indicate significant differences according to the Duncan test ($n = 3$, $p \leq 0.05$).

reduction in plant cell growth, a commonly reported effect of ultrasonic treatment, was further exacerbated by the oxidative burst, leading to a reduction in the protein and sugar contents of the cells, particularly after a 20-second exposure to airborne US. The alteration of protein content by US waves was attributed to the breakage of chains, modification of side groups of amino acids, and modification of the protein structure³⁹.

The increase of cytosolic Ca^{2+} might trigger a Ca^{2+} depletion in the vacuole and cell wall, weakening the Ca^{2+} binding pectic polysaccharides capacity, increasing cytoplasmic calcium concentration, and ultimately leading to cell death⁴⁰.

In the food industry, the US is usually used in the modifications of polysaccharide functionalities and their degradation⁴¹. The plant cell wall is a sophisticated composite consisting of polysaccharides, phenolic compounds, and proteins, and is responsible for the cell mechanical strength⁴². The total chemical content of tobacco cells' walls increased after exposure to the US, mainly due to a significant increase in the ferulic-, cinnamic-, p-coumaric-, caffeic acids, lignin, and HA compounds. The formation of cross-links between certain wall proteins and pectin-bound phenolic acids has also been manifested by cell wall stiffening enhancement and the resulting decrease in cell growth⁴³. A remarkable increase in EGase activity in these cells was accompanied by a drastic reduction of pectin and the other flexible components, HB. These alterations in wall materials limited the growth of tobacco cells through the inhibition of cell expansion²⁴.

The increased rigidity of the cell wall, a key find of our research, is a consequence of the peroxidase- (particularly CPO) mediated cross-linking between various substances, including extensin and feruloylated

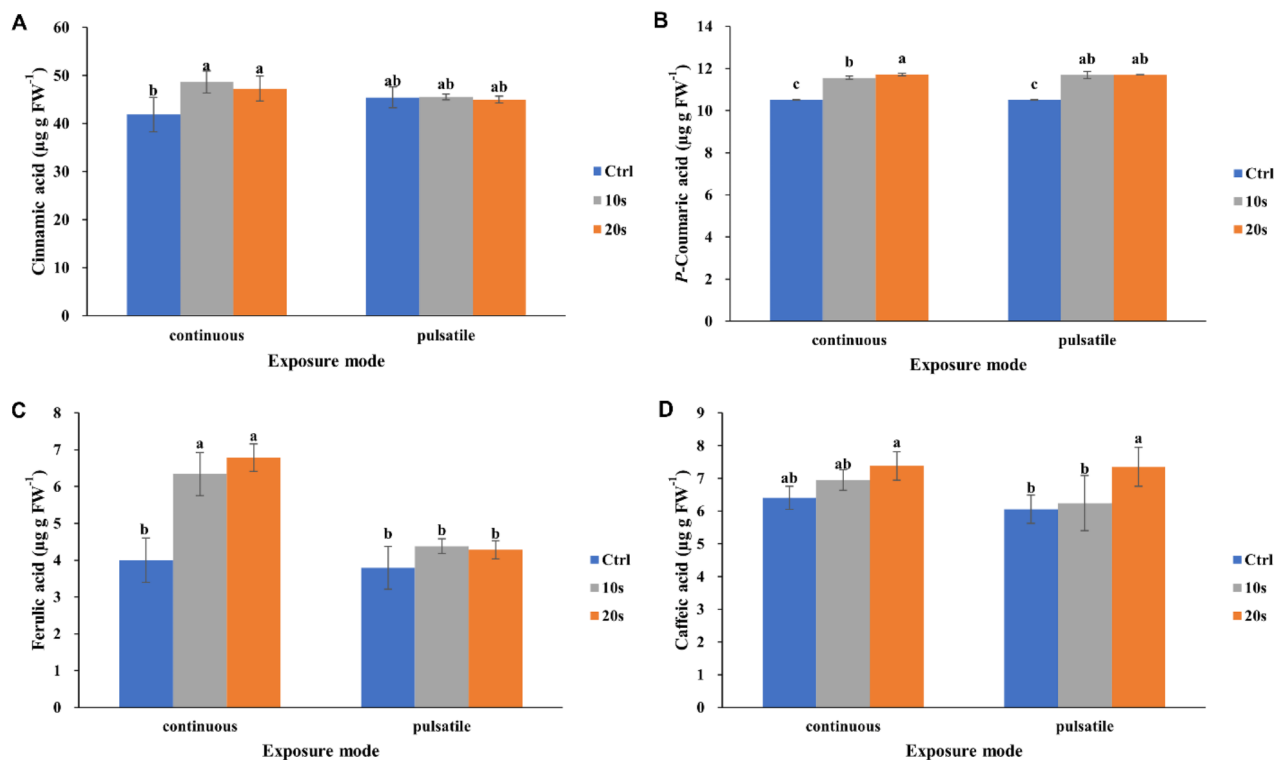


Fig. 7. Effect of airborne US on phenolics metabolism of tobacco cells before and after treatment with 24 kHz airborne US. (A–D) A significant increase in phenolic acids was detected after exposure to both continuous and pulsatile airborne US modes. Different letters indicate significant differences according to the Duncan test ($n = 3, p \leq 0.05$).

polysaccharides, lignin, and phenolic monomers. This cross-linking process, which is catalyzed by peroxidases, is conducted by H_2O_2 as an electron donor. The resulting decrease in extensibility and cell growth can be attributed to this mechanism, which is a key part of the observed effects of ultrasonic exposure on tobacco cells^{43–46}.

The effects of US exposure on peroxidase activity have been previously reported in other studies^{37,47,48}. Wall-bound peroxidases are wall modifying enzymes that restrict cell expansion by catalyzing the formation of cross-links between phenolic acids and polysaccharides¹⁸. The decrease in growth of continuously airborne US-treated tobacco cells in the present study was accompanied by a significant increase in CPO activity and an increase in ferulic acid monomers and lignin polymer.

Conclusion

In conclusion, the results presented here indicate that exposure of plant cells to airborne US, even for a short time, increases the cytosolic calcium concentration, modifies cell wall ultrastructure and enzyme activities, reduces cell growth, and ultimately results in cell death. The effects of the continuous mode of airborne US on the physiology of tobacco cells were more prominent than the pulsatile mode. Considering the energy delivery, the intensity of pulsatile airborne US was lower than the continuous mode, explaining the different effects each mode caused on the exposed cells.

Thus, one might use the approach to evaluate sound pollution effects on the plant's living status. On the other hand, we believe the promising results presented here shed light on the mechanism involved in the effects of different modes of airborne US on tobacco cells. It can be used to manipulate cell physiology for different protective purposes in a physical and non-chemical manner.

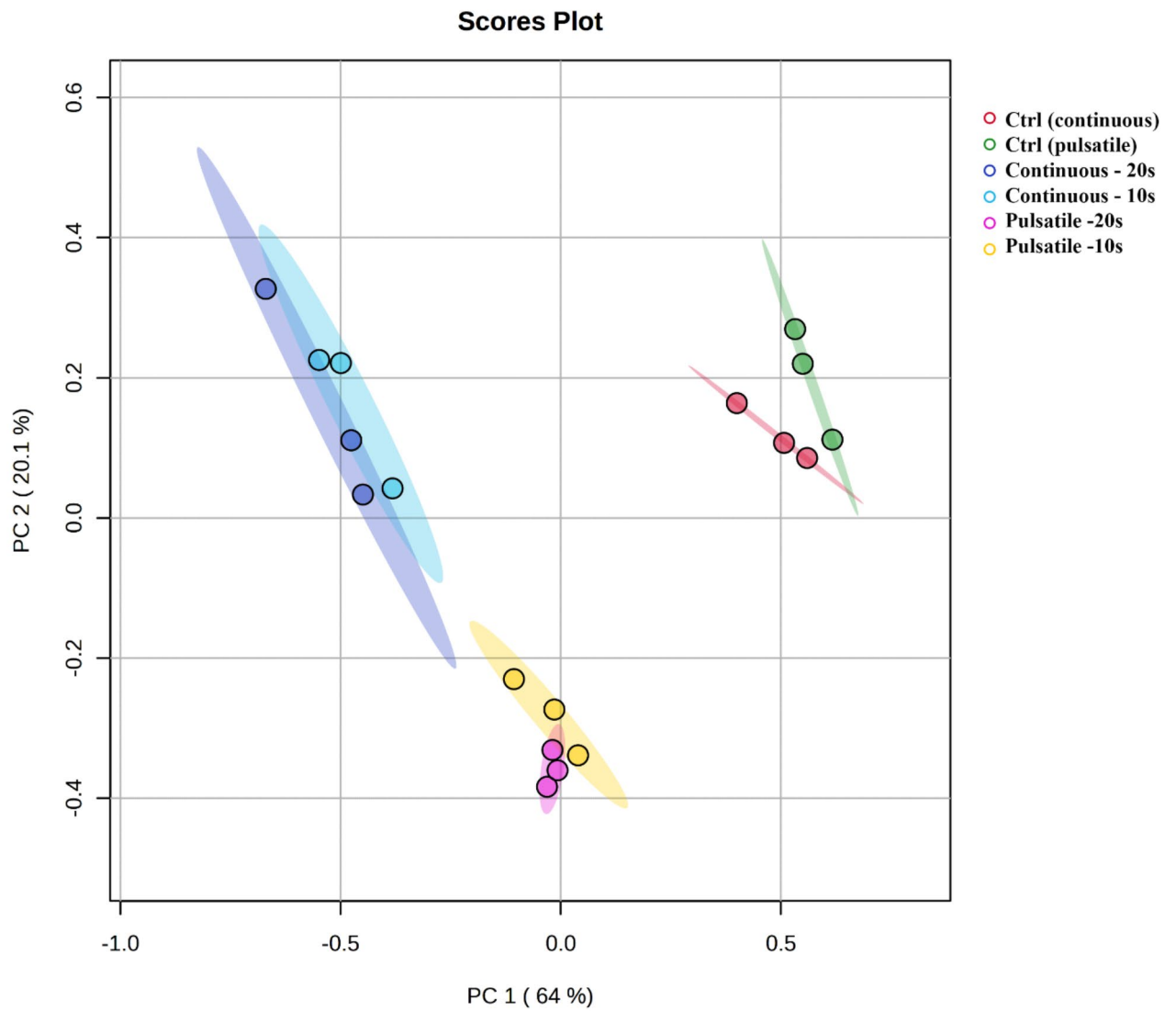


Fig. 8. Correlation among various parameters in airborne US-treated cells. (a) Principal component analysis (PCA).

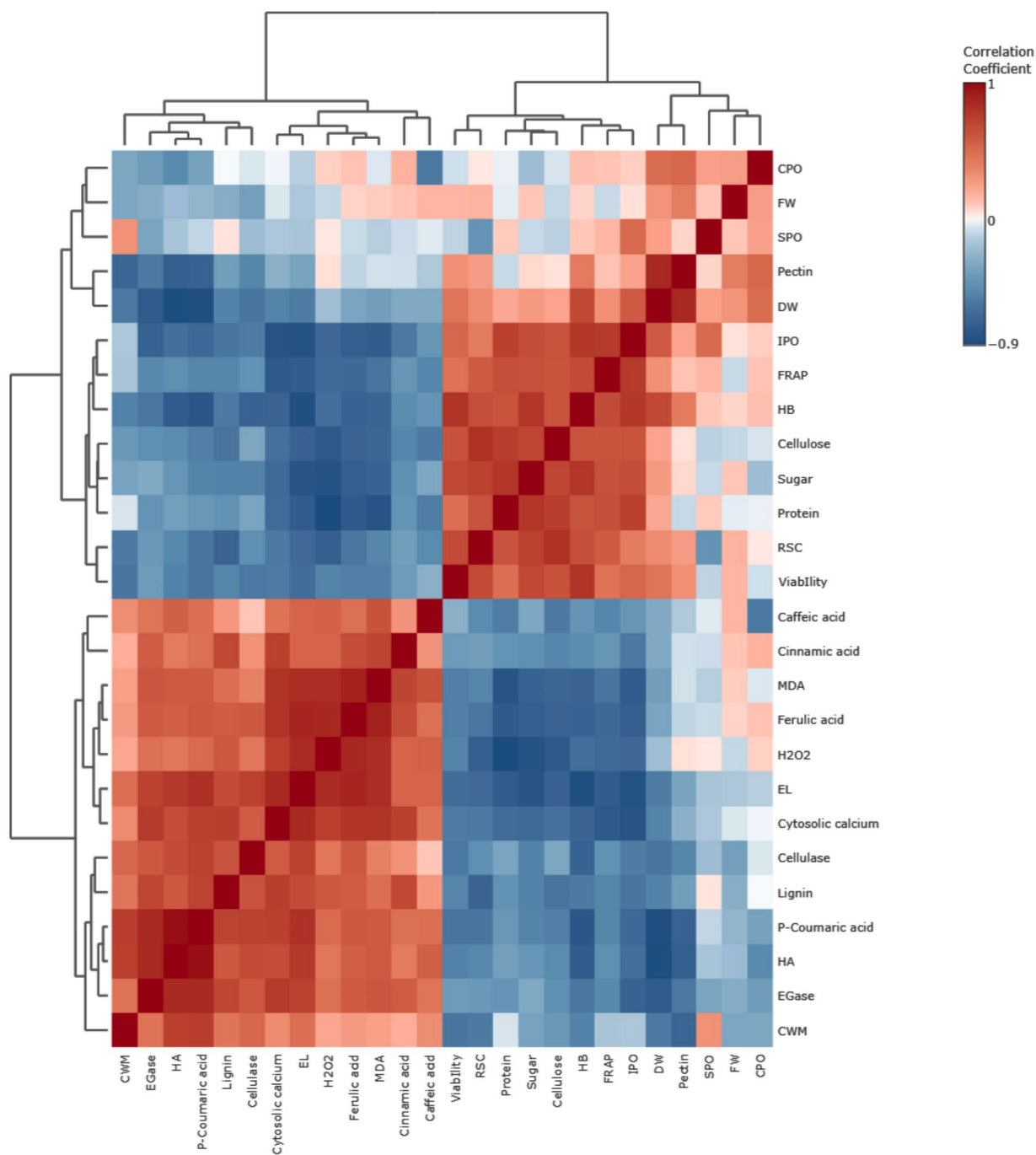


Fig. 9. Heatmap employed for clustering of several parameters according to Pearson correlations coefficient is shown (b) Hierarchical Cluster Analysis (HCA). Data are obtained from three replicates for each variation at all samples. Positive and negative correlations are described by red and blue color, respectively.

Data availability

All data generated or analyzed during this study are included in this manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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