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ORIGINAL ARTICLE

# Sound waves increases the ascorbic acid content of alfalfa sprouts by affecting the expression of ascorbic acid biosynthesis-related genes

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**Abstract** Previous studies have shown that sound wave treatment can affect the expression of plant genes and improve the growth. So, we investigated the ability of sound waves to increase AsA (L-ascorbic acid) content in alfalfa (*Medicago sativa*) sprouts in this study. Sprouts were exposed to a range of sound wave frequencies for two 1-h periods per day for various numbers of days. Most sound wave treated sprouts had a higher AsA content than untreated sprouts. In addition, the activity level of superoxide dismutase, an enzyme with potent antioxidative properties, was increased in sound wave-treated sprouts. The AsA content varied in response to sound wave treatment. Most processing conditions, including 500 and 1000 Hz, increased AsA content by 24–50%; however, some treatment conditions caused reduced AsA content during sprout growth. Furthermore, AsA content during sprout storage was increased by most sound wave treatment conditions, with 13–36% increases observed following 800 and 1000 Hz sound wave treatments compared to untreated sprouts. To investigate the mechanisms underlying changes in AsA content, we analyzed the expression levels of AsA biosynthesis-related genes. We found that several genes, including *VTC1*, *VTC2*, *VTC4*, *GME*, *L-GalDH*, *GLDH*, *MDHAR*, and *DHAR1*, displayed differential expression in response to sound wave treatment. Therefore, sound wave treatment may be a viable method for increasing the nutritional contents of sprouted vegetables.

**Keywords** Alfalfa sprout · Ascorbic acid · Ascorbic acid biosynthesis · Sound wave · Superoxide dismutase · Vitamin C

**Abbreviations**

| | |
|---|---|
| AsA | L-Ascorbic acid |
| VTC1 | GDP-mannose pyrophosphorylase |
| VTC2 | GDP-galactose phosphorylase |
| VTC4 | L-Galactose-1-phosphate phosphatase |
| GME | GDP-D-mannose 3,5-epimerase |
| L-GalDH | L-Galactose dehydrogenase |
| GLDH | L-Galactono-1,4-lactone dehydrogenase |
| MDHAR | Monodehydroascorbate reductase |
| DHAR1 | Dehydroascorbate reductase |

## Introduction

L-Ascorbic acid (AsA), also known as ascorbate or vitamin C, is an antioxidant that plays an important role in plant and animal cellular metabolism. Plants synthesize secondary metabolites such as AsA, phenols, and flavonoids through various biosynthetic pathways. AsA is a major plant antioxidant metabolite (Horemans et al. 2000), which acts as a self-antioxidant molecule by directly removing reactive oxygen species and regenerating α-tocopherol in plant cells (Smirnoff 2000). In addition, AsA regulates hormones that affect plant growth and development, and is involved in other aspects of cellular metabolism involving transcriptome changes

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(Smirnoff et al. 2001). Furthermore, AsA is involved in secondary metabolism pathways, including lipid-soluble α-tocopherol recycling and ethylene biosynthesis, and regulates several fundamental cellular processes, including photoprotection, cell cycle, and cell expansion. AsA is required in the human diet for proper health and nutrition.

Sprouted vegetables, such as bean and alfalfa sprouts, have emerged as a popular food source due to their high nutrient content, favorable texture, and lack of pesticide use during their cultivation. In addition, sprouts have high levels of functional compounds with antioxidant and anticancer properties. Therefore, sprout vegetables are rich in texture and vitamins, and they are appealing to the buyers and they are provided to producers for production process characteristics such as lack of cropland costs, shortened production cycles, human control conditions and industrial production support (Guo et al. 2012). The nutritional content of sprouted vegetables is higher than that in comparable amounts of conventional vegetables, such as alfalfa, broccoli, clover, radish varieties, rapeseed, and cabbage (Song 2001). For example, buckwheat sprouts were found to contain 4.1-fold more rutin, 19-fold more quercetin, and fivefold more chlorogenic acid than the same mass of the conventionally consumed form of mature buckwheat grains (Yoon et al. 2006). In addition, the contents of AsA, phenols, and flavonoids in mung bean sprouts can be increased by irradiation with low-dose UV-B radiation (Wang et al. 2017). Alfalfa sprouts grow rapidly, are a good source of vitamins C, K, and B, and are also a source of saponins that assist in cholesterol balance and support the immune system (Story et al. 1984). Thus, an active area of research concerns investigating the nutritional compounds in sprouted vegetables, with a particular focus on various antioxidant metabolites.

Sound is a vibration of molecules that is transmitted through gases, liquids, or solids. Plants perceive sound, but the underlying mechanisms are poorly understood, and few studies have investigated the ecological, evolutionary, and molecular effects of sound on plants (Gagliano et al. 2012). In addition, there is a lack of research on the effects of sound on plant growth and development, despite recent studies showing that sound waves have the potential to improve plant growth and the quality of plant products. Experiments in chlorella revealed that sound wave treatment significantly promoted chlorella growth and increased the growth rate from 12 to 30% (Jiang et al. 2012). Although it is unclear how a plant can perceive sound, there are a number of known plant sound responses; for instance, promoters have been shown to regulate transgene expression in rice seedlings in response to sound (Jeong et al. 2008) and the directional growth of corn seedling roots has been shown to respond to sound caused by soil-borne vibrations (Gagliano et al. 2012). Sound waves were shown to promote plant growth, seed germination rates, and increase hypocotyl elongation in rice, cucumber, and Arabidopsis seedlings (Takakashi et al. 1992; Johnson et al. 1998; Creath and Schwartz 2004). Sound waves were also demonstrated to enhance root development in paddy rice (Wang et al. 2003a, b) and increase protective enzyme and peroxidase isoenzymes activities in chrysanthemum (Wang et al. 2003a, b). Also in chrysanthemum, it was reported that sound waves regulate the indole-3-acetic acid (IAA) and abscisic acid (ABA) hormones (Bochu et al. 2004). Furthermore, a 1 kHz sound wave delayed tomato fruit ripening by altering the expression of *RIN* and *HB-1*, which are important transcription factors in ethylene biosynthesis (Kim et al. 2015, 2016). Therefore, sound waves are thought to regulate various hormones and gene expression patterns in plants.

AsA is an antioxidant with important metabolic functions in both plants and animals, but the ability to synthesize the compound has been lost in humans and some animals. Thus, consumption of AsA via plants is necessary in the human diet. Although the AsA biosynthetic pathway is well understood in animals, the same pathway in plants has not been characterized. Both the reduced form of AsA (L-ascorbic acid) and the oxidized form (L-dehydroascorbic acid) are necessary for maintaining human health. L-ascorbic acid is converted to L-dehydroascorbic acid in living cells. L-ascorbic acid is synthesized through multiple biosynthetic pathways, including the D-glucosone, D-galacturonate, myo-inositol, and D-Man/L-Gal pathways (Valpuesta and Botella 2004). In the latter pathway, GDP-mannose pyrophosphorylase, encoded by Arabidopsis *VTC1*, provides GDP-mannose, which is used for cell wall carbohydrate biosynthesis, protein glycosylation, and AsA biosynthesis (Conklin et al. 1999). Furthermore, the activity of GDP-L-galactose phosphorylase, encoded by the Arabidopsis paralogous genes *VTC2* and *VTC5*, is an important regulatory mechanism in AsA biosynthesis (Yoshimura et al. 2014). GDP-Man-3′,5′-epimerase (GME) converts GDP-D-mannose to GDP-L-galactose and GDP-L-gulose. Modulation of GME activity by multiple metabolites may be a regulatory mechanism underlying AsA biosynthesis (Wolucka and Van Montagu 2003). AsA activity is reduced in Arabidopsis *VTC4* knockout mutants with defects in L-galactose-1-P phosphatase (Conklin et al. 2006). L-Galactose dehydrogenase (L-GalDH) converts L-galactose-1-phosphate to L-galactono-1,4-lactone (Pignocchi and Foyer 2003; Ishikawa and Shigeoka 2008). L-Galactono-1,4-lactone dehydrogenase (GLDH) converts L-galactono-1,4-lactone to L-ascorbic acid. AsA is reduced by monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR; Yin et al. 2010).

Previous studies have demonstrated that plant gene expression can be artificially regulated by sound wave treatment (Jeong et al. 2008). There has been no report on

the relationship between AsA accumulation and sound wave treatment in plants to date. We hypothesized that the nutrient contents of sprout vegetables, including AsA content, can be increased via optimal sound wave treatment. Thus, we investigated the AsA content and the activities of AsA biosynthesis-related genes in alfalfa sprouts subjected to sound wave treatment.

## Materials and methods

### Plant materials and experimental treatment conditions

Alfalfa (*Medicago sativa*) seeds used in this study were purchased from Danong (Namyangju, Korea), a company specializing in sprouted vegetable seed supply. The seeds were sown in a sprouter (Asia Seed Company, Seoul, Korea) and germinated at 23–25 °C and 45–60% humidity in darkness for 2 days. Following germination, sprouts were cultivated for 5 days under 16 h light/8 h dark conditions. Sprouts were exposed to various sound wave frequencies (250, 500, 800, 1000, and 1500 Hz) for two 1-h periods for various numbers of days. Treated sprouts were sampled in two lots: one sample was stored at 4 °C for subsequent measurement of AsA content and one sample was frozen in liquid nitrogen and stored at − 80 °C for subsequent measurement of superoxide dismutase (SOD) activity. The single-frequency signal was generated using Pro Tools M-Powered software (Avid Technology, USA). The speaker volume was 80 dB. To block external noise, the sound wave treatments were performed in a custom-made, sound-proof chamber (Korea Scientific Technique Industry Co, Korea). The sound level within the growth chamber was approximately 40 dB, whereas the sound level in a commercial growth chamber reaches approximately 80 dB. Sprouts were placed in the sound-proof chambers to prevent the transfer of vibrations between samples during the sound wave treatments. Following treatment, sprouts were placed in a storage room at 23–25 °C and monitored for quality changes. For the control groups, sprouts were exposed to the same experimental conditions except for the sound wave treatments. To investigate the effect of sound waves on sprouts during storage, sprouts cultivated for 6 days were sound wave treated after harvest and stored at 10 °C. Sound wave was treated with sound waves of 250, 500, 800, 1000, and 1500 kHz under twice treatment for 1 h. Sprouts were sampled following storage for 1, 3, and 7 days for analysis of AsA contents, changes in gene expression, and SOD activity.

### Measurement of ascorbic acid content

Samples (2 g) were weighed in a test tube and combined with 20 mL of 5% metaphosphoric acid (Wako, Japan). Samples were homogenized (Ultra-Turrax T25, IKA Labo) and then brought up to a 50 mL total volume. After shaking for 20 min (200 rpm), sample mixtures were extracted with an ultrasonic extractor for 30 min, centrifuged (3200 rpm), and the resulting supernatants were filtered through a 0.45 μm syringe filter. HPLC (NANOSPACE SI-2, SHISEIDO, Japan) was used to detect sample AsA contents. A SHISEIDO Capcell-pak C18 MG120 column was used and column temperature was maintained at 40 °C. The flow rate was 0.5 mL/min, 5 μL of samples was injected, and AsA signals were analyzed at 254 nm using a PDA detector (Thermo Fisher, San Diego, CA USA). A standard solution was prepared by weighing 0.1 g of HPLC-grade AsA (Sigma, St. Louis, MO, USA) into a 100 mL volumetric flask and diluting the solution to final concentrations of 1, 10, 50, and 100 mg/L. A standard calibration curve was prepared using the peak area of the chromatogram obtained in the HPLC analysis as the $y$ value, and the concentration of the standard solution as the $x$ value. The equation of the calibration curve was $y = 13{,}270.9334$ and the correlation coefficient $R^2$ was 1.000, indicating a linear relationship between AsA concentration and peak area. Sample AsA contents were expressed as mg AsA equivalents per 100 g fresh weight sample tissue (mg/100 g). Three biological replicates were analyzed for each experimental treatment.

### RNA extraction and quantitative real-time RT-PCR (qPCR)

Three independent biological samples were produced for each experimental treatment. Directly after sampling, sprouts were frozen in liquid nitrogen and stored at − 80 °C. Frozen tissue was ground into a powder under liquid nitrogen using a mortar and pestle. Total RNA was extracted using a Plant RNeasy Extraction Kit (Qiagen, Hilden, Germany). RNA samples were treated with DNase I (Qiagen, Hilden, Germany), and cDNA was synthesized using amfiRivert Platinum cDNA Synthesis Master Mix (GenDEPOT, South Korea). Quantitative real-time RT-PCR (qPCR) analysis was performed using AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Korea) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The relative mRNA levels were determined by normalizing the PCR threshold cycle number of each target gene with that of the *Actin* reference gene. Three technical replicates were measured for each biological replicate analyzed. The primers used for qPCR analysis are shown in Supplemental Table 1.

### Measurement of superoxide dismutase activity

Alfalfa sprouts were harvested following experimental sound wave treatment, frozen in liquid nitrogen, and stored at − 80 °C. Frozen sprouts were ground under liquid nitrogen and 100 mg of frozen powdered tissue was defrosted in 20 mM potassium phosphate buffer (pH 6.5) on ice. The extracts were clarified by centrifugation at 13,500×*g* for 15 min at 4 °C and SOD activity was measured in the resulting supernatants using a SOD Determination Kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, SOD activity was measured by mixing 220 μL of the SOD Assay Kit-WST reagents with 20 μL of the sample supernatants. Following incubation for 20 min at 37 °C, the absorbance at 450 nm was measured using a microplate reader (μQuan BioTek Instruments, Winooski, USA). The SOD activity value for each experimental treatment was converted to a percentage of the SOD activity measured in the control.

## Results

### The ascorbic acid content of alfalfa sprouts is increased by sound wave treatment

To investigate the impact of sound waves on AsA biosynthesis, alfalfa sprouts during growth were treated with sound waves of 250, 500, 800, 1000, and 1500 kHz for two 1-h treatment by various date treatment conditions. However, the phenotypes of alfalfa sprouts treated with sound waves were not significantly different from those of untreated control. Thus, following treatment, the AsA content of alfalfa sprouts treated during the growth phase was measured using HPLC, which, for most of the experimental samples, revealed increased AsA content as a result of sound wave treatment. In particular, treatment with 1000 Hz sound waves for 2 days resulted in the highest observed increase in AsA content, with levels being approximately 50% more than in untreated samples. Furthermore, 3-day 500 Hz and 6-day 500 Hz sound wave treatments increased AsA content to 40 and 38% more than in the control, respectively. However, alfalfa sprout AsA content was lower as a result of 4- and 6-day 1500 Hz and 5-day 800 Hz sound wave treatment compared to the untreated sample (Table 1). Furthermore, the AsA content in the sprouts was higher following most of sound wave treatments, regardless of whether the treatment was continued for more than 1 day (Table 2). Particularly, the greatest increase in AsA content of approximately 50% was measured in sprouts treated with 500 Hz for 1–6 days. The second highest AsA content increase of approximately 36% resulted from 1000 Hz sound wave treatment for 2–5 days, whereas the third highest increase of approximately 24% was observed following treatment with either 250 Hz sound waves for 1–5 days or 500 Hz sound waves for 2–6 days. Conversely, for treatment with 1500 Hz sound waves, AsA content decreased after treatment for 1–4, 2–5, and 2–6 days (Table 2). These results indicate that the AsA content of alfalfa sprouts can be increased by treatment with specific sound wave frequencies with appropriate timing and for appropriate durations.

### Sound wave treatment affects the expression of ascorbic acid biosynthesis-related genes in alfalfa sprouts

Prompted by the observed increases in AsA content described above (Tables 1, 2), we hypothesized that AsA biosynthesis was affected by sound wave treatment. Therefore, we examined the expression levels of genes related to AsA biosynthesis, namely *VTC1, VTC2, VTC4, GME, L-GalDH, GLDH, MDHAR*, and *DHAR1*, following various sound wave treatments. Expression of these genes was analyzed in alfalfa sprouts at various time points after sound wave treatment with different frequencies, and changes in transcription levels were determined via qPCR analysis using the gene-specific primers listed in Supplemental Table 1. The expression of AsA biosynthesis-related genes increased after each day of treatment for most of the sound wave frequencies compared to untreated samples (Fig. 1). The expression levels of most genes were slightly increased following a 2-day treatment with 1000 Hz sound waves, which was the treatment that resulted in the highest AsA content. Notably, *MDHAR* expression was significantly increased following treatment with 250 and 1000 Hz sound waves. For sprouts treated for 5 days, which displayed the second-highest increase in AsA content, the expression levels of most genes were increased by all sound wave frequencies. *DHAR* and *MDHAR* expression was particularly elevated following the 250 Hz sound wave treatment. Expression levels of *VTC4*, *MDHAR*, and *DHAR1* were increased by the third day of sound wave treatment; however, the expression levels of other genes were similar to those in the untreated control. Conversely, the expression levels of AsA biosynthesis-related genes were largely decreased following 4 and 6 days of the 1500 Hz treatment and 5 days of the 800 Hz sound wave treatment, which were the treatments that also decreased AsA content compared to the control samples. In addition, for sound wave treatments that continued for more than 1 day, the expression levels of most of AsA biosynthesis-related genes were also increased compared to gene expression levels in untreated sprouts (Fig. 2). In particular, following treatment for 1–6 days, which led to the greatest increase in AsA content, expression levels of

**Table 1** Analysis of ascorbic acid content following each day of sound wave treatment in alfalfa sprouts

| | 250 Hz | | 500 Hz | | 800 Hz | | 1000 Hz | | 1500 Hz | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Amount | Value (%) | Amount | Value (%) | Amount | Value (%) | Amount | Value (%) | Amount | Value (%) |
| 1 day | 28.48 ± 0.6 | 127.44 ± 2.70 | 30.12 ± 0.94 | 134.79 ± 3.29 | 27.4 ± 0.78 | 122.63 ± 2.60 | 29.26 ± 1 | 130.91 ± 3.64 | 27.3 ± 0.35 | 122.16 ± 1.21 |
| 2 day | 30.54 ± 1.17 | 135.35 ± 5.25 | 29.77 ± 0.69 | 133.86 ± 2.26 | 24.95 ± 0.49 | 115.21 ± 1.63 | 33.81 ± 0.53 | 150.04 ± 2.11 | 28.74 ± 0.67 | 125.22 ± 1.98 |
| 3 day | 25.63 ± 1.71 | 114.45 ± 7.62 | 31 ± 1.11 | 138.44 ± 4.33 | 26.84 ± 1.39 | 119.83 ± 4.48 | 26.23 ± 2.62 | 117.1 ± 9.76 | 27.39 ± 1.9 | 122.29 ± 7.26 |
| 4 day | 22.45 ± 1.08 | 100.46 ± 4.85 | 28.9 ± 0.63 | 129.3 ± 2.81 | 23.55 ± 0.26 | 105.38 ± 0.89 | 25.62 ± 1.28 | 114.63 ± 5.44 | 22.09 ± 0.53 | 98.85 ± 2.07 |
| 5 day | 24.88 ± 1.75 | 113.96 ± 7.62 | 24.14 ± 0.67 | 107.91 ± 2.92 | 20.57 ± 1.47 | 91.97 ± 6.10 | 22.46 ± 1.36 | 100.59 ± 6.59 | 22.98 ± 1.11 | 102.76 ± 5.18 |
| 6 day | 22.40 ± 0.95 | 100.27 ± 4.27 | 31.74 ± 1.61 | 142.06 ± 7.18 | 29.06 ± 1.27 | 130.06 ± 4.01 | 21.78 ± 1.58 | 97.46 ± 5.44 | 21.19 ± 0.87 | 96.43 ± 4.01 |

The vitamin C content of the control was 22.11 ± 1.08 and the value was calculated in comparison with the control

Standard vitamin C value was expressed in terms of mg ascorbic acid equivalents per 100 g in fresh weight (mg/100 g)

Amount (mg/100 g)

Value (relative value)

**Table 2** Analysis of ascorbic acid content following multiple-day sound wave treatment in alfalfa sprouts

| | 250 Hz | | 500 Hz | | 800 Hz | | 1000 Hz | | 1500 Hz | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Amount | Value (%) | Amount | Value (%) | Amount | Value (%) | Amount | Value (%) | Amount | Value (%) |
| 1–4 days | 25.57 ± 0.30 | 117.72 ± 1.37 | 25.44 ± 0.75 | 117.14 ± 2.93 | 25.13 ± 1.12 | 115.72 ± 4.40 | 25.63 ± 0.40 | 117.98 ± 1.58 | 21.25 ± 0.13 | 97.83 ± 0.51 |
| 2–5 days | 24.86 ± 0.68 | 113.91 ± 3.12 | 22.58 ± 0.76 | 103.46 ± 3.07 | 24.05 ± 1.00 | 110.18 ± 4.45 | 29.83 ± 0.83 | 136.66 ± 3.45 | 19.78 ± 1.98 | 90.62 ± 6.63 |
| 1–5 days | 28.18 ± 1.88 | 124.43 ± 8.28 | 24.88 ± 1.47 | 109.85 ± 5.23 | 22.99 ± 1.46 | 101.53 ± 5.85 | 27.16 ± 0.60 | 119.94 ± 2.62 | 26.03 ± 1.95 | 114.94 ± 7.18 |
| 2–6 days | 25.12 ± 2.02 | 119.42 ± 9.58 | 26.21 ± 2.74 | 124.59 ± 10.91 | 24.16 ± 2.31 | 114.85 ± 8.82 | 21.28 ± 1.64 | 101.17 ± 6.78 | 18.36 ± 1.38 | 87.28 ± 6.49 |
| 1–6 days | 23.96 ± 1.28 | 106.34 ± 5.68 | 34.25 ± 0.63 | 151.99 ± 2.62 | 32.14 ± 2.38 | 142.65 ± 6.96 | 32.66 ± 1.49 | 144.95 ± 4.62 | 27.07 ± 0.73 | 120.15 ± 2.24 |

The vitamin C content of the control was 22.11 ± 1.08 and the value was calculated in comparison with the control

Standard vitamin C value was expressed in terms of mg ascorbic acid equivalents per 100 g in fresh weight (mg/100 g)

Amount (mg/100 g)

Value (relative value)

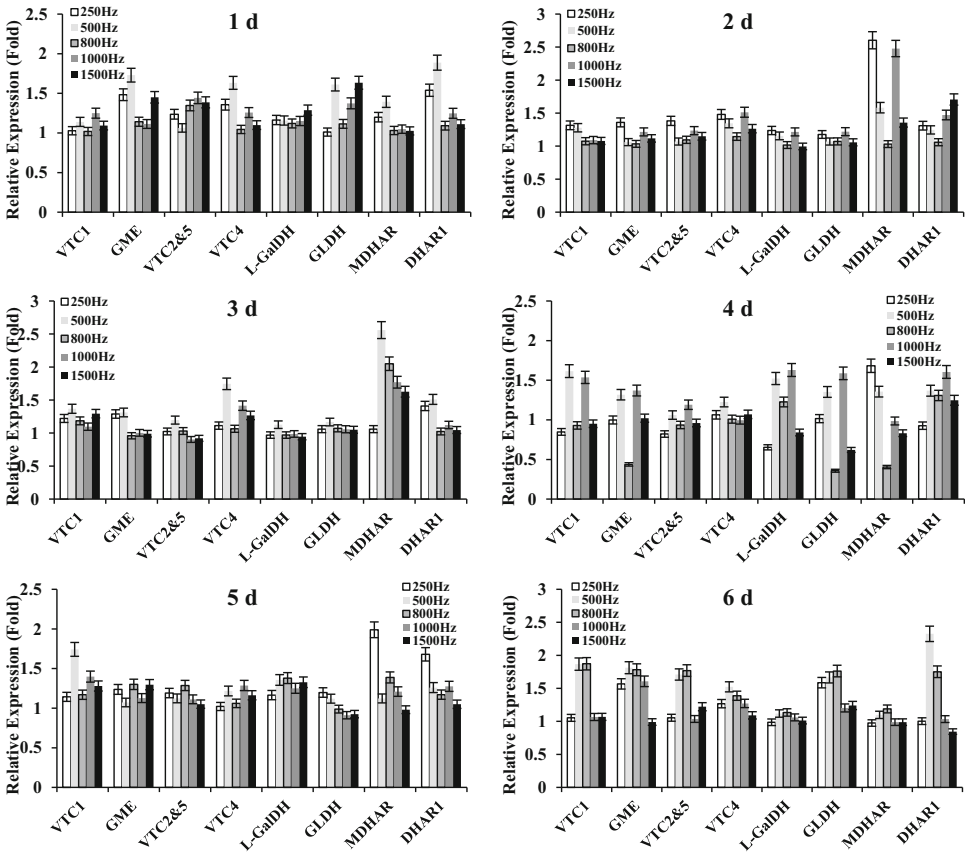


**Fig. 1** Expression of ascorbic acid biosynthetic genes following each day of sound wave treatment in alfalfa sprouts. Expression of ascorbic acid biosynthesis-related genes in alfalfa sprouts at the indicated time points following each day of treatment with 250, 500, 800, 1000, and 1500 Hz sound waves, as determined by qPCR. Error bars indicate SD of three biological replicates. Values are normalized against that in the control

*VTC1* and *GME* were increased by 500 and 1500 Hz sound wave treatments, whereas *VTC4* expression was increased by 1000 and 1500 Hz sound waves, and *MDHAR* and *DHAR1* expression were increased by 500 and 1000 Hz sound waves, respectively. For sound wave treatment for 2–5 days, the expression levels of most genes were increased by 1000 Hz sound waves and the expression levels of *GME* and *DHAR1* were increased by 250 Hz sound waves. Most genes except for *GLDH* and *MDHAR* displayed increased expression levels induced by 250 Hz sound waves, and the expression of a number of genes was increased by 500 and 1000 Hz sound waves. However, comparable to those samples that displayed decreased AsA content following the 1500 Hz sound wave treatment for 1–4 days, most gene expression levels were unchanged or decreased following sound wave treatment for 2–5 days compared to the control samples. For sound wave treatment for 2–6 days, the expression levels of all genes examined except *GLDH* were decreased by treatment with 1500 Hz sound waves. These results indicate that sound wave treatment significantly affects the expression of genes that underlie AsA biosynthesis in alfalfa sprouts, and that each AsA biosynthesis-related gene maintains a specific response to different sound waves.

## The activity of superoxide dismutase is increased by the sound wave treatment in alfalfa sprouts

As increased AsA content in sound wave-treated alfalfa sprouts was observed, we investigated whether the various sound wave treatments also affected SOD activity. SOD is an important enzyme because, like AsA, it has an antioxidant function; specifically, SOD catalyzes the dismutation of superoxide anion with hydrogen peroxide and molecular oxygen. Thus, we measured SOD activity in extracts made from alfalfa sprouts treated with various sound wave conditions. Similar to the changes observed for the expression levels of AsA biosynthesis-related genes and AsA content, SOD activity (inhibition rate) was increased in alfalfa sprouts following sound wave treatment compared to that in untreated control samples (Fig. 3). The greatest increase in SOD activity resulted from the 2-day 1000 Hz sound wave treatment. Also, higher SOD activity was increased by 500 Hz sound wave treatment for the third-day. However, in agreement with the observed decrease in AsA content, SOD activity remained unchanged and was slightly decreased by the 4- and 6-day 1500 Hz sound wave treatment, respectively (Fig. 3a). For treatments that lasted for more than 1 day, the greatest increase in SOD activity was caused by 500 Hz sound wave treatment for 1–6 days

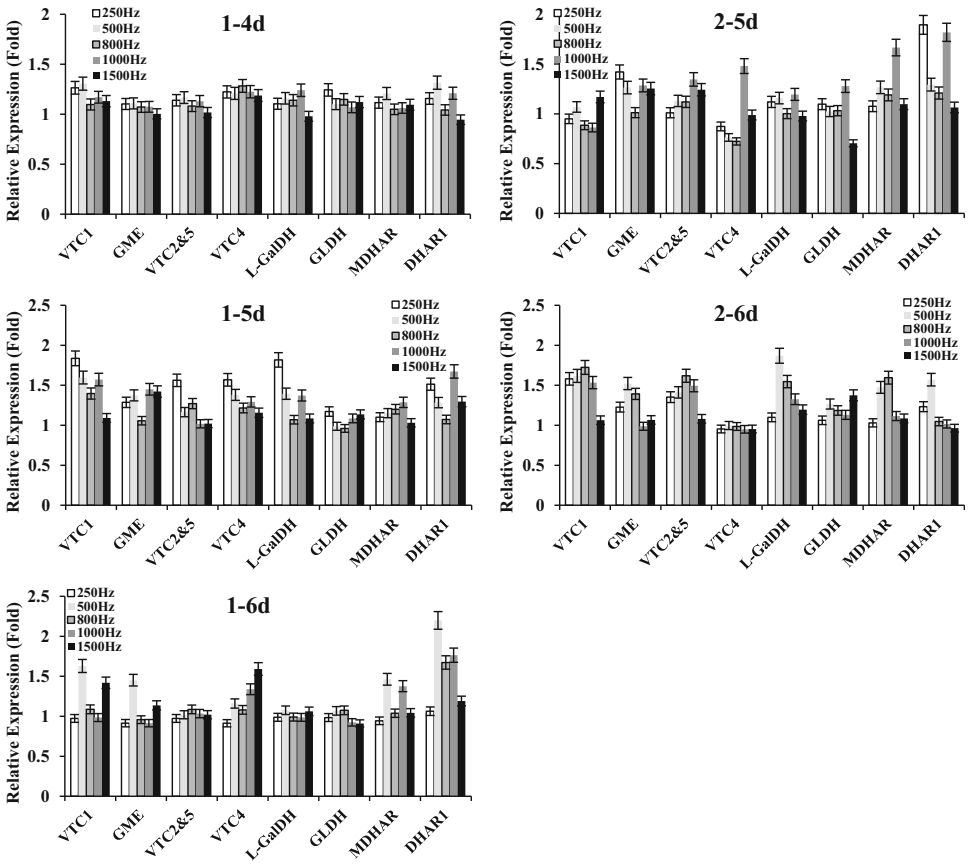


**Fig. 2** Expression of ascorbic acid biosynthetic genes following multiple-day sound wave treatment in alfalfa sprouts. Expression of ascorbic acid biosynthesis-related genes in alfalfa sprouts at the indicated time points following multiple-day treatment with 250, 500, 800, 1000, and 1500 Hz sound waves, as determined by qPCR. Error bars indicate SD of three biological replicates. Values are normalized against that in the control

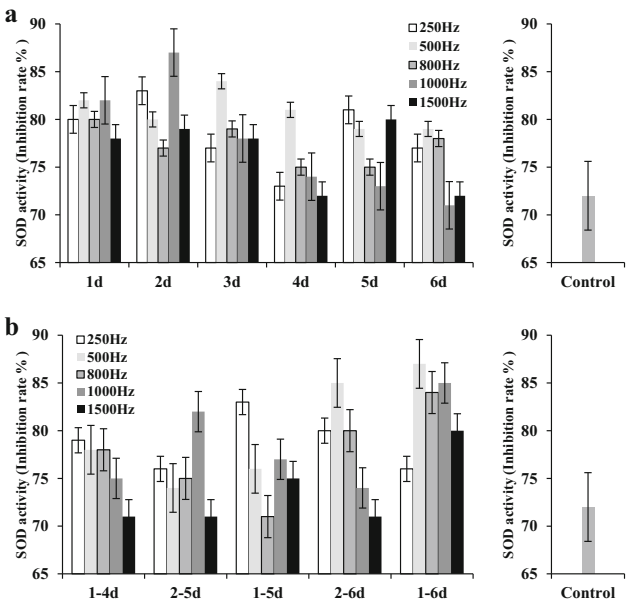


**Fig. 3** Changes in total SOD enzyme activity following sound wave treatment in alfalfa sprouts. **a** Relative SOD enzyme activity following each day of treatment with 250, 500, 800, 1000, and 1500 Hz sound waves. **b** Relative SOD enzyme activity following multiple-day sound wave treatment. Error bars indicate SD of three biological replicates

(Fig. 3b). Also, higher SOD activity was induced by 500 Hz sound wave treatment for 2–6 days and by 250 Hz sound wave treatment for 1–5 days. These results suggest that sound wave treatments in alfalfa sprouts increase the activity of antioxidant enzymes.

## Sound waves increase the antioxidant activity in alfalfa sprouts during storage

In addition to examining the effects of sound wave treatments on antioxidant activity, in particular AsA content, in alfalfa sprouts during the growth phase, we investigated if comparable treatments affected antioxidant activity during storage. Following sound wave treatment, alfalfa sprouts were analyzed after 1, 3 and, 7 day of storage. Ascorbic acid content in stored sprouts was increased by all sound wave treatment conditions compared with the untreated control, with the greatest increase from at least 13 up to 36% being induced by 800 and 1000 Hz sound waves in both 1, 3 and 7 days conditions (Fig. 4a). Similarly, SOD activity in stored sprouts was increased by most sound wave treatments, including 800 and 1000 Hz sound waves (Fig. 4b). Finally, the expression levels of AsA biosynthesis-related genes in stored sprouts were increased following most sound wave treatments compared to those in

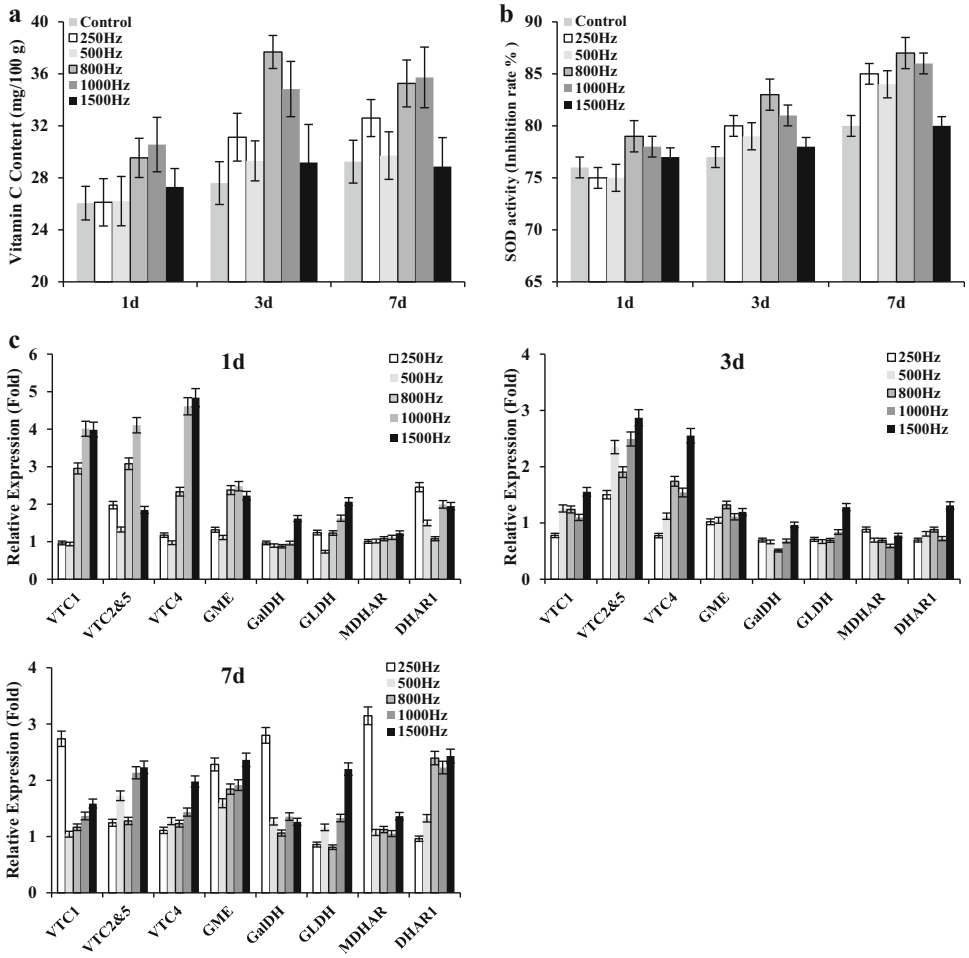


**Fig. 4** Analysis of ascorbic acid content in stored alfalfa sprouts following sound wave treatment. **a** Analysis of ascorbic acid content in stored alfalfa sprouts following treatment with 250, 500, 800, 1000, and 1500 Hz sound waves. **b** Relative SOD activity in stored alfalfa sprouts following sound wave treatment. **c** Expression of ascorbic acid biosynthesis-related genes in stored alfalfa sprouts following sound wave treatment, as determined by qPCR. Error bars indicate SD of three biological replicates. Values are normalized against that in the control

untreated stored sprouts (Fig. 4c). Particularly, the expression levels of *VTC1*, *VTC2*, *VTC5*, *VTC4*, and *GME* measured after 1 day of storage following treatment with 800–1500 Hz sound waves were higher than the expression levels of other genes. After 3 days of storage, *VTC2*, *VTC5*, and *VTC4* expression levels were increased and *VTC1*, *GME*, *L-GalDH*, and *MDHAR* expression levels were increased after 7 days of storage following a 250 Hz sound wave treatment. These results indicate that, as during the growth period, sound wave treatment enhances antioxidant activity in alfalfa sprouts during storage and, particularly, increases the AsA content of stored sprouts by influencing the expression levels of AsA biosynthesis-related genes.

## Discussion

In this study, we investigated the potential of using sound wave treatment to increase the content of AsA, a functional antioxidant, in alfalfa sprouts. Sprout AsA content was analyzed following treatment with various sound wave frequencies. AsA is a micronutrient necessary for maintaining the function and health of the human body, and is synthesized through multiple biosynthetic pathways. However, whereas plants and most animals can synthesize AsA internally, humans and other certain animal species cannot, and therefore must consume AsA in their diet. AsA has been a research focus for many years, and studies have been performed attempting to increase the amounts of various nutritional substances, including AsA, in sprouted vegetables. Plant seeds are rich in the nutrients required to support biological processes and growth. Sprouted vegetables, which are young seedlings harvested within a week following seed germination, have a shorter cultivation period and, because they produce many substances to protect themselves from the external environment during early growth, contain more vitamins, minerals, and functionally active substances than mature vegetables.

Here, we tested the effects of various single sound wave frequencies and multiple treatment periods on sprouts. For both short- and long-term treatment, sprout AsA content was increased in response to sound waves compared to untreated sprouts. AsA content was increased by approximately 38–50% following short-term sound wave treatment

and by approximately 24–50% following long-term sound wave treatment. However, certain sound wave treatments decreased sprout AsA content. In addition, higher AsA content was observed in harvested alfalfa sprouts stored at 10 °C following sound wave treatment than in untreated stored sprouts. Therefore, we propose that AsA content in alfalfa sprouts is affected by the sound wave treatment, growth phase, and treatment period.

Sound waves applied at different frequencies, pressure levels, exposure times, and distances from the source have been shown to affect plant growth. Thus, acoustic biology is emerging as a research focus and sound waves should be considered when examining the impact of environmental stress on plant growth and development. Previous studies have reported that sound vibrations stimulate seed germination and plant growth (Weinberger and Burton 1981). In addition, yield and quality in crops such as tomato, barley, and vegetables have been improved using sound waves (Hou and Mooneyham 1999; Spillane 1991; Xiao 1990). For example, treatment with 1 kHz sound waves delayed fruit ripening in tomato by regulating the expression of the major ethylene biosynthesis transcription factors *RIN* and *HB-1* (Kim et al. 2015, 2016). In an additional previous study, transgenic rice containing *Alcohol dehydrogenase* (*Ald*) promoter–GUS constructs displayed significantly increased GUS expression following 125, 250, and 1 kHz sound wave treatment. By contrast, these plants contained significantly reduced levels of GUS transcript following 50 Hz sound wave treatment, suggesting that the *Ald* promoter responds to sound waves in a frequency-specific manner (Jeong et al. 2008). In addition, in chrysanthemum, specific sound wave treatments (1.4 kHz, 95 dB, 10 days) have been reported to regulate plant hormones promoting an increase in IAA and a decrease in ABA (Bochu et al. 2004). Sound stimulation can also enhance plant disease resistance and reduce the need for chemical fertilizer and pesticide use (Zhang 2012). These studies have led to the conclusion that sound waves affect various plant hormones and gene regulation. However, despite the demonstrated potential of sound wave treatment to improve plant growth and development, experimental evidence describing the effects of sound wave treatment in plants is severely limited.

In this study, we demonstrate that sound wave treatment contributes to an increase in AsA content in alfalfa sprouts by influencing AsA biosynthesis-related genes. The AsA content in alfalfa sprouts was measured by HPLC following various sound wave treatments applied during sprout growth, which revealed increased AsA content in response to most sound wave conditions compared to the untreated sprouts. Therefore, we investigated expression changes in AsA biosynthetic genes in sound wave-treated alfalfa sprouts. In the AsA biosynthetic pathway, GDP-D-mannose generated from D-mannose 1-phosphate is sequentially converted to GDP-L-galactose, L-galactose 1-phosphate, L-galactose, L-galactono-1,4-lactone, and lastly L-ascorbic acid (AsA; Valpuesta and Botella 2004). *VTC1*, *VTC2*, *VTC5*, *VTC4*, *L-GalDH*, and *GLDH* are involved in the biosynthesis of L-ascorbic acid, the reduced form of vitamin C, and *MDHAR* and *DHAR1* are involved in the biosynthesis of L-dehydroascorbic acid, the oxidized form of vitamin C (Conklin et al. 1999, 2006; Wolucka and Van Montagu 2003; Yoshimura et al. 2014; Pignocchi and Foyer 2003; Yin et al. 2010). We observed increased expression levels for most AsA biosynthesis-related genes following sound wave treatment compared to gene expression levels in untreated sprouts. In particular, *MDHAR* and *DHAR1* expression increased more dramatically in response to sound treatment than that of other genes during sprout growth. These results suggest that sound wave treatment affects the expression of AsA biosynthetic genes, which in turn increases the AsA content in alfalfa sprouts. Since AsA is a major antioxidant, we examined SOD activity to investigate whether sound wave treatment affects other antioxidant sources in plants. Comparable to the observed increases in AsA content, SOD activity in alfalfa sprouts was higher following sound wave treatment, suggesting that sound waves affect antioxidant activity in plants in general. In addition to investigating the effects of sound wave treatment during sprout growth, we analyzed AsA content and the activity of other antioxidants in stored sprouts following sound wave treatment. AsA content, expression levels of AsA biosynthesis-related genes, and other antioxidant activity in stored sprouts were also enhanced by sound wave treatment. These results indicated that the AsA content in alfalfa sprouts may be increased via sound wave treatment either during sprout cultivation or distribution. However, the increase of vitamin C content and the expression of major biosynthetic genes by sound wave treatments seem to require treatment with appropriate conditions and specific sound frequency. Therefore, the correlation between increased AsA content by sound wave treatment and the expression levels of major AsA biosynthetic genes should be studied in more detail.

In conclusion, the results of our study showed that the AsA content of alfalfa sprouts can be increased by sound wave treatment, and that the sound wave frequency, treatment time, and treatment period influence final AsA levels. Further research is needed to identify the optimal sound wave treatment to achieve the most desirable AsA levels. The results of study are expected to be applicable in the production of sprout vegetables with high contents of functional materials. The outcomes of this study support the conclusions of previous research that it is possible to influence the expression of plant genes, which induces

various physiological changes, by sound wave treatment. Therefore, we expect that future systematic research will lead to sound wave treatment technology being applied in agriculture as well as in other industries.

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