

Sound waves affect the total flavonoid contents in *Medicago sativa*, *Brassica oleracea* and *Raphanus sativus* sprouts

Joo Yeol Kim, Ye Eun Kang, Soo In Lee, Jin A Kim, Muthusamy Muthusamy and Mi-Jeong Jeong^{*} 



Abstract

BACKGROUND: Sound waves are emerging as a potential biophysical alternative to traditional methods for enhancing plant growth and phytochemical contents. However, little information is available on the improvement of the concentration of functional metabolites like flavonoids in sprouts using sound waves. In this study, different frequencies of sound waves with short and long exposure times were applied to three important varieties to improve flavonoid content. The aim of this study was to investigate the effect of sound waves on flavonoid content on the basis of biochemical and molecular characteristics.

RESULTS: We examined the effects of various sound wave treatments (250 Hz to 1.5 kHz) on flavonoid production in alfalfa (*Medicago sativa*), broccoli (*Brassica oleracea*) and red young radish (*Raphanus sativus*). The results showed that sound wave treatments differentially altered the total flavonoid contents depending upon the growth stages, species and frequency of and exposure time to sound waves. Sound wave treatments of alfalfa (250 Hz), broccoli sprouts (800 Hz) and red young radish sprouts (1 kHz) increased the total flavonoid content by 200%, 35% and 85%, respectively, in comparison with untreated control. Molecular analysis showed that sound waves induce the expression of genes of the flavonoid biosynthesis pathway, which positively corresponds to the flavonoid content. Moreover, the sound wave treatment significantly improves the antioxidant efficiency of sprouts.

CONCLUSIONS: The significant improvement of flavonoid content in sprouts with sound waves makes their use a potential and promising technology for the production of agriculture-based functional foods.

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INTRODUCTION

Vegetable sprouts are a rich source of vitamins, minerals, amino acids and bioactive substances and are known as a healthy human diet worldwide. More importantly vegetable sprouts are rich in flavonoids, well-known plant-based antioxidants, and vitamins A, C and E.¹ These antioxidants and minerals act as immune modulators and have various biological activities including anti-inflammatory, anticancer, antiviral and antiaging properties.² Nonetheless, consistent efforts are taking place among plant researchers and dieticians to improve the nutritional profiles of vegetable sprouts, through alteration of environmental factors influencing bioactive molecules such as external signals. A recent study illustrates that artificial light sources and temperature conditions can influence the accumulation of physiologically active substances in vegetable sprouts.³ Flavonoids are diverse plant pigments functioning in stress resistance and as safe non-immunogenic drugs that prevent cardiovascular diseases and memory loss, and restore skin damage related to UV rays. Flavonoids are derived from phenylalanine, which is converted to anthocyanins or other flavonoids⁴ via flavonoid

biosynthetic enzymes in the cytoplasm. Newly formed flavonoids are transported to the vacuole or cell wall.⁵ Structural genes encoding the major enzymes in the flavonoid pathway and the regulatory genes necessary for flavonoid biosynthesis have been characterized in various plant species.⁵

Plants respond to biotic and abiotic stimuli, as well as to external signals such as light, wind and sound.⁶ Spontaneous and artificially generated sound waves affect various mechanisms related to physiological responses, plant growth and development.^{6,7} In the past, Gagliano *et al.*⁸ showed that root growth in germinating maize (*Zea mays*) seeds was directed by sound waves. Literature evidence shows that sound waves

^{*} Correspondence to: M-J Jeong, Department of Agricultural Biotechnology, National Institute of Agricultural Science, Rural Development Administration, 370 Nongsaengmyeong-ro, Deokjin-gu, Jeonju, Jeollabuk-do, 54874, Republic of Korea. E-mail: center1097@korea.kr

Department of Agricultural Biotechnology, National Institute of Agricultural Science, Rural Development Administration, 370 Nongsaengmyeong-ro, Deokjin-gu, Jeonju, Jeollabuk-do, 54874, Republic of Korea

can also alter phytohormone levels via regulation of hormone synthesis-related genes in plants.^{9–12} In *Chrysanthemum* spp. sound waves increased the concentration of growth hormone indole acetic acid and decreased the level of another phytohormone, abscisic acid.⁹ Moreover, it is clear that sound waves alter the expression level of gibberellic acid, salicylic acid and jasmonic acid biosynthesis-related genes of *Arabidopsis thaliana*.¹⁰ Sound waves at a frequency of 1 kHz were previously used to delay the ripening process in tomato (*Solanum lycopersicum*) fruit by regulating the expression of genes encoding transcription factors involved in ethylene biosynthesis.^{11,12} Reports also show that sound waves improve the radical scavenging efficiency of peroxidase isoenzymes and other enzymes with antioxidative properties in *Chrysanthemum* spp.¹³ However, sound waves with different frequencies can differentially regulate gene expression and plant growth as evidenced in the past, where high-frequency sound waves (125 Hz, 250 Hz or 1 kHz) increased the promoter activities of alcohol dehydrogenase while low-frequency sound (50 Hz) decreased them.¹⁴ From these studies, it is clear that sound waves with specific frequencies will alter the physiological and biochemical content of plants by differentially regulating biosynthesis pathway-related genes.

In the study reported herein, we investigated the total flavonoid content in three economically important vegetable sprouts with sound waves of low to high frequencies and with different exposure times. We demonstrated that the flavonoid contents could be altered by sound wave treatments. Furthermore, we identified the best sound wave frequency, plant growth stage and treatment duration for increasing the flavonoid content in these vegetable sprouts.

MATERIALS AND METHODS

Plant materials and treatment conditions

Seeds of the three vegetables used in this study, alfalfa (*Medicago sativa*), broccoli (*Brassica oleracea*) and red young radish (*Raphanus sativus*), were purchased from Danong Co. (Namyangju, Korea), a company specializing in vegetable sprout seeds. The seeds were sown in a sprouter (Asia Seed Company, Seoul, Korea) and germinated at 23 °C and 45–60% humidity for 2 days under dark conditions. Following germination, the sprouts were cultivated at 23 °C for 5 days under 16 h light/8 h dark conditions (light intensity radiation: 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Sprouts were exposed to various sound wave treatments. Single frequencies of 250 Hz, 800 Hz, 1 kHz and 1.5 kHz were used to analyze the effects of sound waves. In this study, 250 Hz was considered as low frequency, 800 Hz as moderate frequency and 1 and 1.5 kHz as high frequencies. The sprouts were placed in soundproof chambers (Korea Scientific Technique Industry Co, Korea) to prevent the transfer of vibrations between samples during the treatments (Fig. 1(A)). The speaker was a GENELEC 8010A (GENELEC, Lisalmi, Finland) with a frequency response is 74 Hz to 20 kHz. The background noise in the growth chamber without sound wave treatment was approximately 40 dB and the sound level reached by sound wave treatment was approximately 80 dB in this experiment. In order to select the best sound treatment conditions, sprout responses to different sound wave treatments were investigated, such as sound wave frequency, growth stage and total sound wave exposure time (Fig. 1(B)). For short-term (ST) treatments, sprouts were treated daily for 1 h each in the morning and afternoon on days 2 and 4 after sowing (termed ST2 and ST4, respectively). For long-term (LT) treatments, sprouts were treated for 1 h each in the morning and afternoon at 1 to

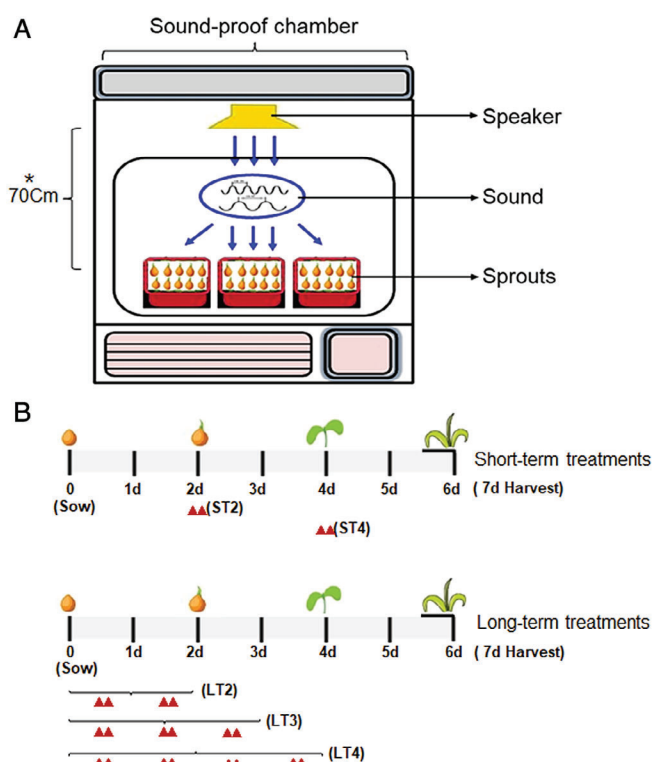


Figure 1. Schematic diagram of the sound wave treatment experiment used to analyze flavonoid levels in vegetable sprouts. (A) The soundproof chamber and method used to produce sound waves. (B) The sound wave treatment periods. Red triangles represent a 1 h sound wave treatment. Asterisk (*) indicates the distance between the speaker and the plants.

2 days, 1 to 3 days, and 1 to 4 days after sowing (termed LT2, LT3 and LT4, respectively). For the untreated control groups, sprouts were exposed to the same conditions but without exposure to sound waves. The treated sprouts were sampled at 7 days after sowing, frozen in liquid nitrogen and stored at -80°C .

Quantification of total flavonoid content

As a prerequisite to quantify flavonoids, a standard calibration curve was constructed using diluted naringin (Sigma, St Louis, MO, USA) with concentrations of 0, 25, 50, 100, 200 and 400 mg L^{-1} . Amounts of 500 μL of naringin in diethylene glycol (JUNSEI, Japan) and 500 μL of 1 mol L^{-1} NaOH (Sigma, St Louis, MO, USA) were mixed thoroughly using a shaker for 1 h at 37 °C and the absorbance was measured at 420 nm using a microplate reader (μQuan BioTek Instruments, Winooski, VT, USA). The absorbance values were plotted on the y-axis and naringin concentration on the x-axis. The gross dose rate was calculated as $y = 336.33x - 6.4315$, and the correlation coefficient (R^2) was 0.9986, indicating good linearity.

The experimental samples were prepared with 100 mg of tissues homogenized in 1 mL of 50% methanol (JUNSEI, Japan) vortexed for 15 s, and shaken at 200 rpm for 16 h at room temperature. The mixtures were centrifuged at $1000 \times g$ for 20 min at room temperature and the supernatant (650 μL) was transferred to a new 2 mL tube and the centrifugation step was repeated again. A 500 μL aliquot of each supernatant sample was combined with 5 mL of diethylene glycol and 500 μL of 1 mol L^{-1} NaOH in a 15 mL tube and shaken at 37 °C for 1 h. Total flavonoid content was measured at 420 nm and expressed as milligrams of flavonoid per

liter of sample. The final concentration represents the average derived from three biological replicates for each treatment.

RNA extraction and quantitative RT-PCR

Three independent biological samples were produced for each experimental treatment. Immediately after sampling, the sprouts were frozen in liquid nitrogen and stored at -80°C . The frozen tissue was ground into a powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using a Plant RNeasy Extraction Kit (Qiagen, Hilden, Germany). The RNA samples were treated with DNase I (Qiagen, Hilden, Germany), and cDNA was synthesized using amfiRivert Platinum cDNA Synthesis Master Mix (GenDEPOT, Barker, TX, USA). Quantitative RT-PCR (qPCR) analysis was performed using AccuPower 2X GreenStar qPCR Master Mix (Bioneer, Korea) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Relative mRNA levels were determined by normalizing the PCR threshold cycle number of each target gene with that of the *Actin* reference gene. In qPCR analysis, three technical replicates were measured for each biological replicate analyzed. The primers used for qPCR analysis are presented in the supporting information (Table S1).

2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical assay

The DPPH radical assay was performed as described by Kim *et al.*¹⁵ Each $20\ \mu\text{L}$ extract sample was combined with $180\ \mu\text{L}$ of $0.2\ \text{mmol L}^{-1}$ DPPH (Sigma, St Louis, MO, USA) dissolved in ethanol (JUNSEI, Japan) and adjusted to a final volume of $200\ \mu\text{L}$. The mixtures were vigorously shaken and incubated for 30 min at room temperature in the dark. Absorbance was measured at 515 nm using a microplate reader. Neutralization of the DPPH radical was calculated using $S(\%) = 100 \times (A_0 - A_s)/A_0$, where A_0 is the absorbance of the control (containing all reagents except the test compound) and A_s is the absorbance of the sample. Three technical replicates were measured per biological replicate.

FRAP assay

FRAP reagent was freshly prepared by mixing 25 mL of acetate buffer ($300\ \text{mmol L}^{-1}$, pH 3.6) (Sigma, St Louis, MO, USA), 2.5 mL of TPTZ solution (10 mL of TPTZ in $40\ \text{mmol L}^{-1}$ HCl) (Sigma, St Louis, MO, USA) and 2.5 mL of $20\ \text{mmol L}^{-1}$ FeCl_3 (Sigma, St Louis, MO, USA) in water. A $10\ \mu\text{L}$ aliquot of each extract dissolved in the appropriate solvent was added to $300\ \mu\text{L}$ of FRAP reagent in each well of a 96-well plate and incubated for 30 min at 37°C . Absorbance was measured at 593 nm using a microplate reader. All experiments were performed in triplicate, and all results are presented as the Trolox equivalent antioxidant capacity. Trolox (Sigma, St Louis, MO, USA) standard solution at concentrations of 0, 5, 10, 20, 40 and $80\ \text{mmol L}^{-1}$ was prepared and the absorbance was measured at 593 nm to construct a standard calibration curve, where absorbance was plotted on the y-axis and the concentration on the x-axis. The gross dose rate was $y = 0.0503x + 0.0387$, and the correlation coefficient (R^2) was 0.9998, indicating good linearity.

Statistical analysis

An analysis of variance (ANOVA) was performed using the Statistical Package for Social Science (SPSS, version 25.0, IBM Corporation, NY, USA), and a Duncan's multiple range test was used to determine the statistical significance of the means at $P < 0.05$.

RESULTS AND DISCUSSION

Sound wave treatment alters total flavonoid contents in vegetable sprouts

To improve the biosynthesis of key secondary metabolites in plants, several factors including the cultivation environment can be optimized.¹⁶ Although several studies of increasing physiologically active substances in plants (including secondary metabolites) in various growth environments have been reported, few studies have focused on increasing functional compound levels in vegetable sprouts using sound wave treatments.^{17,18} In this study, we selected the best sound wave treatment conditions for increasing total flavonoid contents in alfalfa, broccoli and red young radish sprouts by varying the sound frequency, growth stage and total sound wave exposure time. The sprouts were subjected to 1 h ST treatments in the morning and afternoon or 1 h LT treatments in the morning and afternoon on multiple days after sowing.

The results showed that total flavonoid content of the three vegetable sprouts used in this study was altered in response to sound wave frequencies and exposure times (Table 1). For alfalfa sprouts, sound waves at a frequency of 250 Hz or 1.5 kHz in ST2 or ST4 treatments increased the total flavonoid content (Table 1(A)). Sprouts treated with 250 Hz sound waves in ST showed maximum total flavonoid content, i.e. 200–215% relative to the untreated control for all treatments. Under LT treatment, the greatest increase in total flavonoid content, i.e. *ca* 200% increase relative to the untreated control, was observed in response to 250 or 800 Hz under LT2 (Table 1(A)). It is clear that a 1- or 2-day sound wave treatment increased the total flavonoid content in alfalfa sprouts more than long exposure treatments (3 days or longer). Overall, these observations show that the growth stage after sowing and the sound frequency have strong effects on total flavonoid contents in vegetable sprouts.

For broccoli sprouts, the increase in total flavonoid content was highest in the 250 Hz treatment group versus the other conditions under ST treatment, with the highest increase observed with ST4 at 250 Hz (*ca* 27% increase; Table 1(B)). The second highest increase under ST treatment occurred for 250 Hz ST2 samples, with levels approximately 13% higher than those of the untreated control. Under LT treatment, the total flavonoid contents increased *ca* 35% in 800 Hz LT4 samples, and they increased *ca* 30% in 1 kHz LT3 plants (Table 1(B)). In sprouts treated at the same frequency of 800 Hz, the highest content was observed in LT4, and the lowest in LT2. These results suggested that the growth stage and exposure time have a significant impact on total flavonoid content. In addition, in broccoli sprouts, the total flavonoid content decreased in the LT2 treatment regardless of the sound frequency during the early stage.

The total flavonoid content in red young radish sprouts increased in response to all ST sound wave treatments, and the increase was especially strong in the 1 kHz ST4 treatment. The ST4 treatment increased total flavonoid contents *ca* 85%, representing the highest increase among treatments. The next highest increase rate was *ca* 60% in response to 250 Hz ST2 and ST4 treatments (Table 1(C)).

Among the vegetable sprouts tested, sound wave treatments were most effective at increasing total flavonoid content in alfalfa, especially for the 250 Hz and 800 Hz LT2 treatments. For broccoli sprouts, the 800 Hz LT4 and 1 kHz LT3 treatments were best, and for red young radish sprouts, the 1 kHz ST4 treatment was the best condition. Total flavonoid content was higher under LT than ST treatment for alfalfa and broccoli sprouts while ST treatment produced higher flavonoid content in red young radish sprouts, especially for 250 Hz ST2 and ST4 treatments, suggesting the

Table 1. Analysis of total flavonoid content after short-term and long-term sound wave treatments in (A) alfalfa, (B) broccoli and (C) red young radish sprouts. The untreated control amounts (values) are 2.5 ± 0.7 (100 ± 28.6)^{cd}, 51.7 ± 1.5 (100 ± 2.9)^{gh} and 117.6 ± 1.9 (100 ± 1.6)^g for alfalfa, broccoli and red young radish, respectively. The numerical amounts represent mg L^{-1} and the value was calculated in comparison with the control. Each value represents the mean of three measurements ($\pm$ SE). Different letters indicate significantly different values ($P < 0.05$) calculated using ANOVA followed by a Duncan's multiple range test

(A) Alfalfa								
	250 Hz		800 Hz		1 kHz		1.5 kHz	
	Amount	Value (%)	Amount	Value (%)	Amount	Value (%)	Amount	Value (%)
ST2	5.3 ± 1.5^i	213.7 ± 60.5^i	3.3 ± 0.8^{ef}	133.7 ± 33.6^{ef}	2.6 ± 0.7^d	103.8 ± 26.9^d	3.9 ± 0.8^{gh}	157.6 ± 33.6^{gh}
ST4	5.4 ± 0.1^i	214.5 ± 40.3^i	2.9 ± 0.8^d	115.8 ± 33.6^d	2.5 ± 1.0^{bcd}	100.1 ± 40.4^{bcd}	4.0 ± 1.2^{fgh}	161.4 ± 47.1^{fgh}
LT2	7.4 ± 1.2^k	297.4 ± 47.1^k	7.4 ± 1.4^k	295.2 ± 53.8^k	5.8 ± 1.0^j	231.6 ± 40.4^j	3.7 ± 0.7^{fg}	147.2 ± 26.9^{fg}
LT3	2.4 ± 0.8^{bc}	95.4 ± 33.6^{bc}	2.1 ± 0.9^b	82.0 ± 33.6^b	4.0 ± 0.8^h	161.2 ± 33.6^h	3.3 ± 1.4^e	133.6 ± 53.8^e
LT4	2.9 ± 0.7^e	115.1 ± 26.9^e	1.6 ± 1.0^{ab}	62.1 ± 40.4^{ab}	1.9 ± 0.7^{ab}	77.6 ± 26.8^{ab}	3.6 ± 0.5^{gh}	145.8 ± 20.2^{gh}
(B) Broccoli								
	250 Hz		800 Hz		1 kHz		1.5 kHz	
	Amount	Value (%)	Amount	Value (%)	Amount	Value (%)	Amount	Value (%)
ST2	58.6 ± 3.7^k	113.3 ± 7.2^k	51.3 ± 3.0^{fg}	99.2 ± 5.9^{fg}	50.3 ± 2.5^{ef}	97.3 ± 4.9^{ef}	52.8 ± 2.5^{hi}	102.1 ± 4.9^{hi}
ST4	66.1 ± 6.0^m	127.9 ± 11.6^m	53.6 ± 3.6^j	103.6 ± 6.9^j	57.5 ± 4.6^k	111.3 ± 8.9^k	48.9 ± 2.8^e	94.5 ± 5.3^e
LT2	35.3 ± 1.0^a	68.2 ± 1.9^a	36.2 ± 1.5^a	70.0 ± 2.9^a	47.4 ± 2.2^d	91.6 ± 4.2^d	44.5 ± 2.5^c	86.2 ± 4.9^c
LT3	51.6 ± 2.7^e	99.8 ± 5.2^e	47.6 ± 2.2^d	92.1 ± 4.2^d	66.3 ± 2.2^l	128.3 ± 4.2^l	41.8 ± 2.9^b	80.8 ± 5.5^b
LT4	53.3 ± 1.4^{hi}	103.2 ± 2.6^{hi}	70.4 ± 1.5^n	136.1 ± 2.9^n	53.7 ± 1.0^l	103.9 ± 1.9^j	53.7 ± 1.4^i	103.8 ± 2.6^i
(C) Red young radish								
	250 Hz		800 Hz		1 kHz		1.5 kHz	
	Amount	Value (%)	Amount	Value (%)	Amount	Value (%)	Amount	Value (%)
ST2	193.5 ± 3.7^s	164.6 ± 5.3^s	141.2 ± 3.0^m	120.1 ± 4.3^m	169.4 ± 3.3^p	144.1 ± 4.9^p	171.3 ± 3.5^q	145.7 ± 3.7^q
ST4	190.1 ± 3.0^f	161.7 ± 2.7^f	137.4 ± 3.0^l	117.4 ± 5.7^l	218.4 ± 5.0^t	185.8 ± 3.3^t	147.1 ± 3.2^n	125.1 ± 5.3^n
LT2	110.9 ± 2.9^e	94.4 ± 4.0^e	100.5 ± 2.4^b	85.5 ± 4.3^b	102.9 ± 2.2^c	87.5 ± 3.3^c	94.3 ± 3.0^a	80.2 ± 2.3^a
LT3	105.5 ± 2.5^d	89.7 ± 3.7^d	149.8 ± 2.4^o	127.4 ± 4.0^o	113.6 ± 1.5^f	96.7 ± 4.7^f	100.8 ± 2.0^b	85.7 ± 5.0^b
LT4	118.4 ± 1.4^h	100.7 ± 2.0^h	120.3 ± 1.5^i	102.3 ± 2.3^i	122.5 ± 1.9^j	104.2 ± 2.0^j	124.2 ± 1.4^k	105.6 ± 3.0^k

impact of sound wave treatment on flavonoid content might differ with vegetable types. Interestingly, in red young radish sprouts, the total flavonoid content decreased in response to prolonged sound wave treatment under LT treatments, perhaps due to stress. It may be a mechanism that is activated differently depending on the structure, cell, tissue and type of plant. However, the exact mechanism is not yet clearly known. Our previous studies have shown that the same gene in rice may increase or decrease in expression depending on the sound frequency.¹⁴ In addition, there were best sound frequencies for the regulation of gene expression related to ethylene biosynthesis of tomato and ascorbic acid biosynthesis of alfalfa sprout vegetable.^{11,18} Moreover, a recent report suggested that four different types of cells when treated with the same sound wave condition responded differently and the expression of related genes also differed.¹⁹ Therefore, because the growth, development and morphology are different in plants, it is estimated that the expression of related genes for a particular sound wave will change differently. Therefore, the effect of sound wave treatment on flavonoid content may vary depending on the type of vegetable.

Similarly, the effect of sound waves on antioxidative enzyme activity differed among various cultivars and tissues of *Debriobium candidum*, supporting our results that the antioxidant defense system was stimulated by sound waves.²⁰ Thus, in addition to flavonoids, the levels of antioxidants could also be increased in plants treated with sound waves.

Sound wave treatment affects the expression of flavonoid biosynthesis-related genes in vegetable sprouts

Plants exposed to a variety of mechanical perturbations, such as wind and touch, undergo physiological and developmental changes that enhance resistance to subsequent mechanical stress.²¹ These physiological and developmental changes result from rapid and dramatic fluctuations in gene expression; studies have revealed increases in stress resistance in plants via the regulation of stress-related genes, which also influence plant growth and metabolism. We reasoned that plants perceive sound waves as a type of external signal similar to biotic or abiotic stress stimuli. One response to such stimuli is the biosynthesis of secondary metabolites, including ascorbic acid, flavonoids and other compounds that might have protective effects (i.e. antioxidant or antimicrobial activities). Indeed, the concentrations of these compounds increase in response to external stimuli such as light, UV radiation, temperature, ozone, heavy metals and drought conditions.^{22,23}

Changes in total flavonoid contents in response to sound wave treatment likely occur due to the altered expression of genes involved in flavonoid biosynthesis. So, to investigate whether sound wave treatment would increase the total flavonoid content of vegetable sprouts by altering the expression of flavonoid biosynthesis genes, we analyzed the expression of 11 flavonoid biosynthesis-related genes in sound wave-treated samples using qPCR. The expression of these genes was analyzed by selecting three higher and one lower conditions of total flavonoid contents

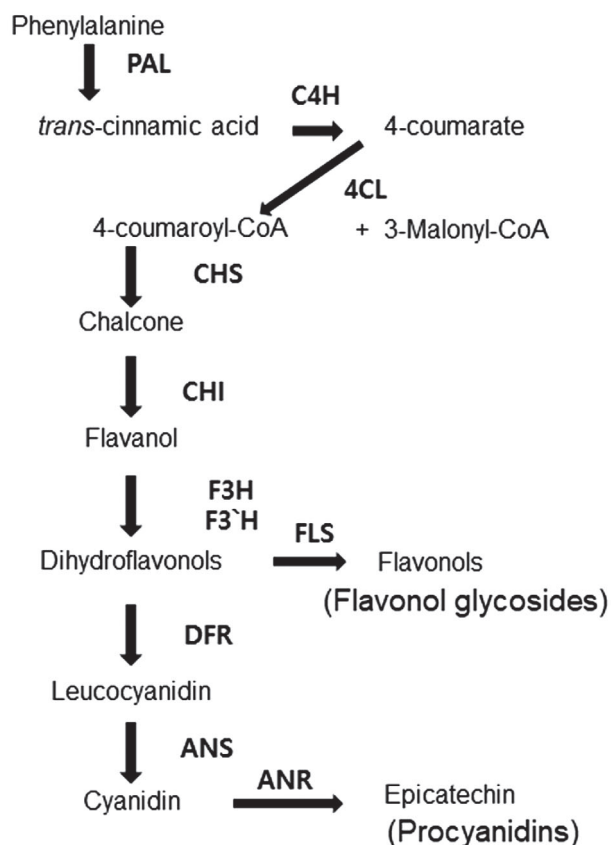


Figure 2. Schematic diagram of general flavonoid biosynthetic pathways. Group I (PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid: CoA ligase). Group II (ACC, acetyl-CoA carboxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase). Group III (DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase).

in the three vegetable sprouts. The genes can be classified into three major groups, i.e. general phenylpropanoid pathway-related genes (*PAL*, *C4H* and *4CL*); flavonoid biosynthesis-related genes (*CHS*, *CHI*, *F3H*, *F3'H* and *FLS*); and other genes regulating flavonoid biosynthesis (*DFR*, *ANR* and *ANS*) (Fig. 2).

PAL, *C4H* and *4CL*

PAL transcript level increased in alfalfa sprouts under the 250 Hz ST4 treatment, representing the highest increase under ST treatment (Fig. 3(A)). The *C4H* transcript was upregulated under the 800 Hz LT2 treatments. However, all three transcripts were down-regulated in response to 1 kHz LT4 treatment, which was reflected by a decrease in total flavonoid content (Fig. 3(A)). In broccoli sprouts, the *PAL* and *C4H* transcripts were upregulated in response to the 250 Hz ST4 treatment, which also resulted in the highest total flavonoid content among the ST treatments. Similarly, *PAL* and *C4H* transcripts were also upregulated in response to the 800 Hz LT4 and 1 kHz LT3 treatments, while they were down-regulated under the 800 Hz LT2 treatment (Fig. 3(B)). All three genes were upregulated in red young radish sprouts under the 250 Hz ST2, 1 kHz ST4 and 800 Hz LT3 treatments, where the total flavonoid content increased (Fig. 3(C)). However, under the 1.5 kHz LT2 treatment condition, where the total flavonoid content decreased, expression of *4CL*, *PAL* and *C4H* decreased. Thus, it is clear that the expression pattern of *PAL* and *C4H* is a key factor

determining the total flavonoid content in broccoli and red young radish sprouts. In other words, changes in *PAL* and/or *C4H* (but not *4CL*) expression were related to changes in total flavonoid content. This result indicated that *PAL*, *C4H* and *4CL* expressions were related to changes in flavonoid content in alfalfa, broccoli and red young radish sprouts.

CHS, *CHI*, *F3H*, *F3'H* and *FLS*

The expressions of *CHS*, *CHI*, *F3H* and *F3'H* genes were induced in alfalfa sprouts in response to 250 Hz ST4, 250 Hz LT2 and 800 Hz LT2 treatments, positively corresponding the increase in total flavonoid content (Fig. 4(A)). On the other hand, the transcript levels of all five genes were reduced in response to the 1 kHz LT4 treatment, reflecting the low increase in flavonoid content. Genes other than *FLS* were expressed at higher levels under the LT than ST treatment, especially *CHI* and *F3'H*. In broccoli sprouts, all five gene expressions were higher under three treatment conditions (250 Hz ST4, 800 Hz LT4 and 1 kHz LT3) in which total flavonoid content increased (Fig. 4(B)). Indeed, the high accumulation of flavonoid content at 800 Hz LT4 is mainly due to higher level of gene expression. Similar to alfalfa, the changes in gene expression and changes in total flavonoid content are correlated. All five gene expressions were higher in red young radish sprouts under the same conditions that produced an increase in total flavonoids (250 Hz ST2, 1 kHz ST4 and 800 Hz LT3; Fig. 4(C)). Therefore, the expression levels of these genes increased when total flavonoid contents increased in vegetable sprouts in response to sound wave treatment. So, these results indicate that the specific sound waves seem to cause an increase the expression of flavonoid biosynthesis-related genes, leading to increased flavonoid content in sprouts.

DFR, *ANR* and *ANS*

Among the three genes *DFR*, *ANR* and *ANS*, *DFR* expression was upregulated in alfalfa sprouts under 250 Hz ST4, 250 Hz LT2 and 800 Hz LT2 treatments that increased the total flavonoid content (Fig. 5(A)). However, under the 1 kHz LT4 treatment, which resulted in a decrease in total flavonoid content, *ANR* expression was higher than untreated control even though the expression of genes was lower. Similarly, *DFR*, *ANR* and *ANS* expression was higher in broccoli sprouts under 250 Hz ST4, 800 Hz LT4 and 1 kHz LT3 conditions (Fig. 5(B)). However, under the 800 Hz LT2 treatment, which decreased the total flavonoid content, *DFR* and *ANS* expression decreased, but *ANR* expression was higher as in alfalfa. So, *ANR* expression does not seem to be related to flavonoid content variation. *DFR* expression was higher in red young radish sprouts under conditions that increased the total flavonoid content (250 Hz ST2, 1 kHz ST4 and 800 Hz LT3), but under the 1.5 kHz LT2 treatment, for which the total flavonoid content decreased, *ANS* expression was higher (Fig. 5(C)).

Previous studies reported that upregulated expression of flavonoid biosynthesis pathway genes was responsible for accumulation of various flavonoid subclasses in radish.²⁴ *CHS*, *CHI*, *F3H*, *F3'H* and *DFR* were also more strongly upregulated than the other genes in response to increasing frequency sound wave treatment in all vegetable sprouts (Figs 3–5), corroborating the variation in total flavonoid content. Whereas the expression levels of these genes were lower in sprouts showing reduced total flavonoid content for specific sound wave treatments; the expression levels of *PAL*, *C4H* and *4CL*, as well as *FLS*, *ANS* and *ANR* were not significantly regulated than flavonoid biosynthesis-related genes

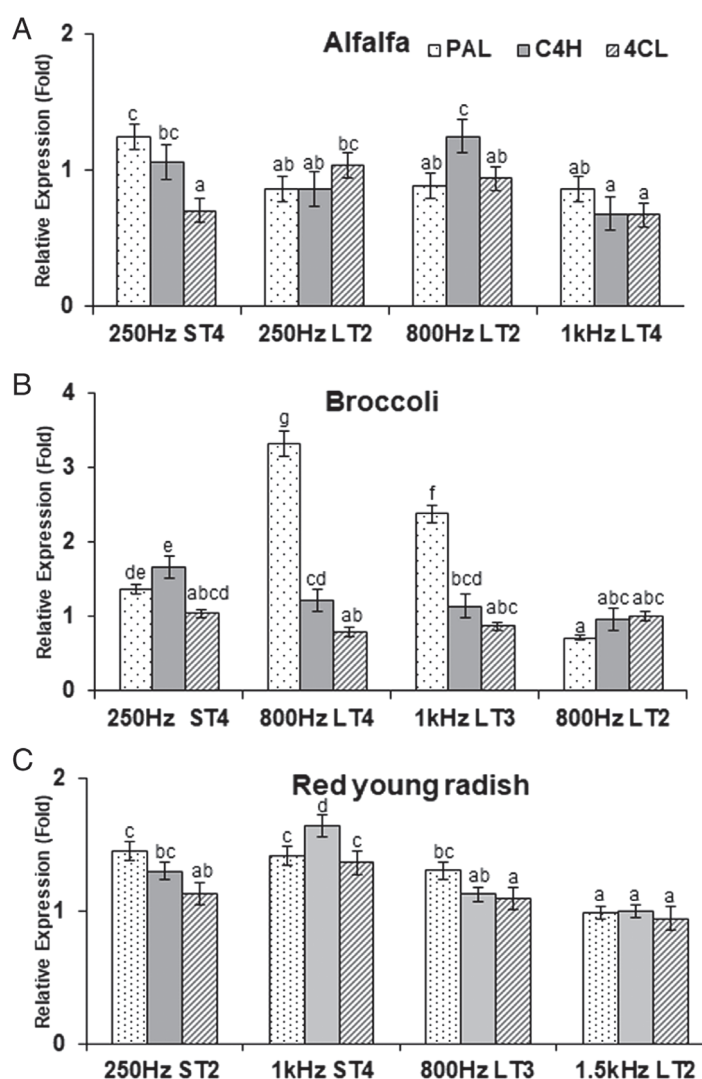


Figure 3. Expression patterns of general phenylpropanoid pathway genes after sound wave treatment in vegetable sprouts. Expression patterns of general phenylpropanoid pathway genes in (A) alfalfa, (B) broccoli and (C) red young radish sprouts after sound wave treatment under the indicated conditions, as determined by qPCR. Error bars indicate SE of three biological replicates. Values are normalized against untreated control. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using ANOVA followed by a Duncan's multiple range test.

by sound wave treatment (Figs 3–5). Thus, the increase in total flavonoid content following sound wave treatment is likely due to the induced expression of key flavonoid biosynthesis genes.

DPPH radical scavenging activity increases in vegetable sprouts in response to sound wave treatment

We investigated whether the antioxidant activity of vegetable sprouts would increase in response to sound wave treatment using the DPPH assay, as antioxidant activity affects the ability of plants to scavenge DPPH radicals. Under ST treatments, the DPPH radical scavenging rate of alfalfa sprouts was higher for sound wave frequencies ranging from 250 Hz to 1.5 kHz regardless of the stage but according to the total flavonoid content. Alfalfa sprouts have lower flavonoid content than broccoli and red young radish sprouts under normal conditions. Indeed, the basic DPPH radical scavenging rate was also lower in these sprouts. Under the 250 Hz ST2 and ST4 treatments, the DPPH radical scavenging rate was approximately 8% higher than the untreated control (Table 2(A)). Moreover, the DPPH radical scavenging rate increased by more

than 10% in sprouts under the 250 and 800 Hz LT2 treatments (Table 2(B)).

The DPPH radical scavenging rate in broccoli sprouts increased under the ST conditions. Similar to alfalfa sprouts, scavenging activity increased in broccoli sprouts under all 250 Hz treatments. A significant increase (10%) in activity was observed for the 250 Hz ST4 treatment (Table 2(A)). For the LT treatments, the highest DPPH radical scavenging rate (*ca* 18% increase) was detected for the 800 Hz LT4 treatment followed by the 1 kHz LT3 treatment (Table 2(B)).

In red young radish sprouts, similar to total flavonoid contents, the DPPH radical scavenging rate was higher in response to all ST treatments (Table 2). The highest DPPH radical scavenging rate (*ca* 11% increase) was found for the 1 kHz ST4 treatment while the second highest rate was reported for 250 Hz ST2. Under LT treatment, the DPPH radical scavenging rate tended to decrease under most conditions, but for 800 Hz LT3, the rate showed little increase (Table 2(B)).

Thus, the pattern of DPPH radical scavenging activity in the vegetable sprouts was similar to that of total flavonoid content.

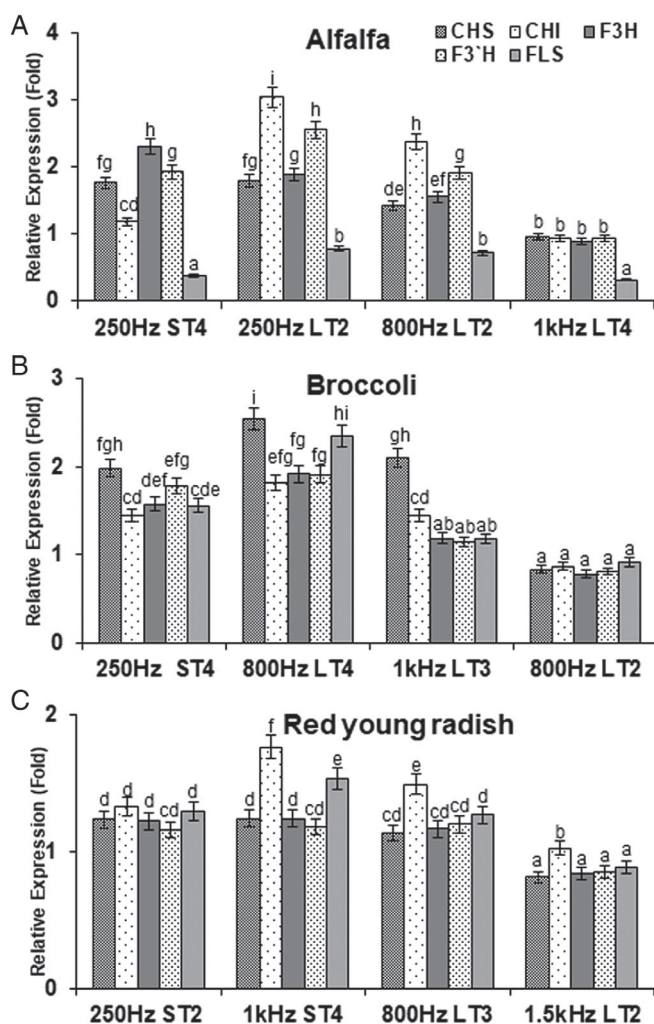


Figure 4. Expression patterns of early flavonoid biosynthesis-related genes after sound wave treatment in vegetable sprouts. Expression patterns of early flavonoid biosynthesis-related genes in (A) alfalfa, (B) broccoli and (C) red young radish sprouts after sound wave treatment under the indicated conditions, as determined by qPCR. Error bars indicate SE of three biological replicates. Values are normalized against untreated control. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using ANOVA followed by a Duncan's multiple range test.

Therefore, the increase in DPPH radical scavenging activity was possibly related to the increase in total flavonoid content during sound wave treatment. Previous reports showed that the total phenolic compound content and DPPH radical scavenging activity are related.²⁵ Hashidoko²⁶ suggested that flavonoids and vitamin C exhibit high radical scavenging ability, as these compounds are active antioxidants. Consequently, these results showed that sound waves increase the flavonoid content, which in turn improves the total antioxidant capacity.

FRAP values increase in vegetable sprouts in response to sound wave treatment

We also investigated the effects of sound wave treatment on vegetable sprouts using the FRAP antioxidant activity assay. In alfalfa sprouts, the FRAP values were higher in response to sound wave treatments than the untreated control. These

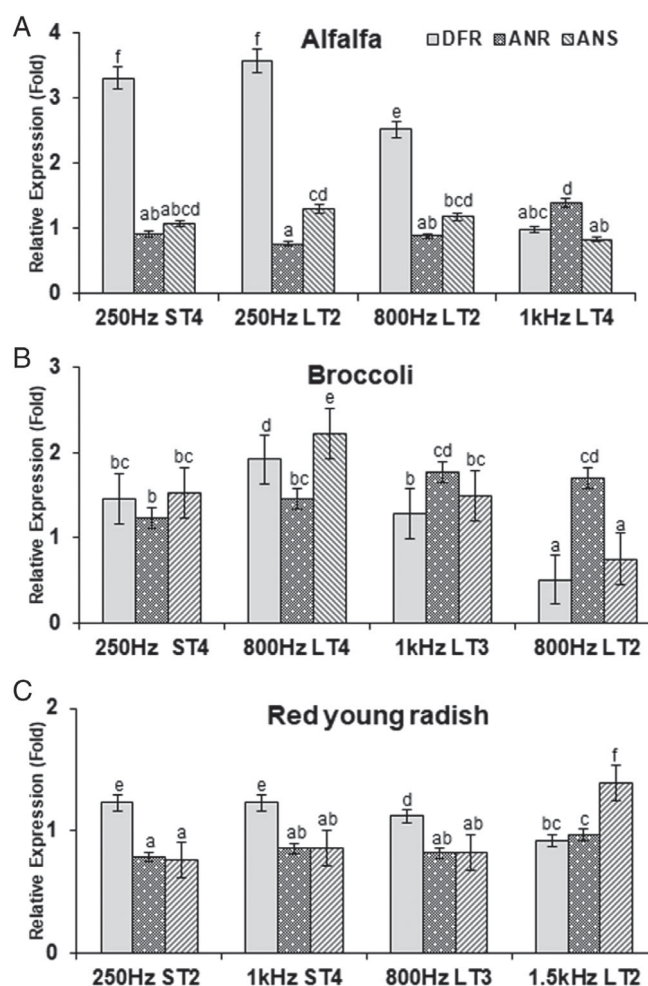


Figure 5. Expression patterns of late flavonoid biosynthesis-related genes after sound wave treatment in vegetable sprouts. Expression patterns of late flavonoid biosynthesis-related genes in (A) alfalfa, (B) broccoli and (C) red young radish sprouts after sound wave treatment under the indicated conditions, as determined by qPCR. Error bars indicate SE of three biological replicates. Values are normalized against untreated control. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using ANOVA followed by a Duncan's multiple range test.

results further support our findings that an increase in total flavonoid content is likely to enhance the antioxidative properties in vegetable sprouts in response to sound waves. The highest FRAP value ($5.52 \text{ mmol L}^{-1} \text{ TE/100 mg}$) was measured in sprouts treated with 250 Hz ST4, i.e. more than twice that of the untreated control (Table 3(A)). Under LT treatment, the highest FRAP values (6.49 and $6.26 \text{ mmol L}^{-1} \text{ TE/100 mg}$) were detected in response to the 250 and 800 Hz LT2 treatments, respectively (Table 3(B)).

The highest FRAP value detected in broccoli sprouts was $15.68 \text{ mmol L}^{-1} \text{ TE/100 mg}$ after the 250 Hz ST4 treatment, and the second highest was measured during the 250 Hz ST2 treatment (Table 3(A)). The FRAP values in broccoli sprouts under the LT2 treatment tended to decrease compared to the untreated control. A high FRAP activity ($16.43 \text{ mmol L}^{-1} \text{ TE/100 mg}$) was observed in broccoli sprouts under the 1 kHz LT3 and 800 Hz LT4 treatments (Table 3(B)).

Table 2. Analysis of DPPH radical scavenging activity after short- and long-term sound wave treatment in alfalfa, broccoli and red young radish sprouts. (A) DPPH radical scavenging activity after short-term sound wave treatment. (B) DPPH radical scavenging activity after long-term sound wave treatment. The values represent the inhibition rate (%). The untreated control values are 55.4 ± 1.71^d , 64.67 ± 1.33^e and 82.03 ± 2.81^e for alfalfa, broccoli and red young radish, respectively. Each value represents the mean of three measurements ($\pm$ SE). Different letters indicate significantly different values ($P < 0.05$) calculated using ANOVA followed by a Duncan's multiple range test

(A)					
Treatment	Sprout	Frequency			
		250 Hz	800 Hz	1 kHz	1.5 kHz
ST2	Alfalfa	63.9 ± 1.73^l	58.63 ± 1.85^g	56.37 ± 1.71^e	60.6 ± 1.85^i
	Broccoli	71.9 ± 3.04^i	64.5 ± 1.99^e	62.57 ± 1.06^d	64.83 ± 5.66^e
	Red young radish	92.13 ± 2.35^k	85.47 ± 3.25^g	91.6 ± 3.12^{jk}	91.4 ± 2.87^j
ST4	Alfalfa	63.57 ± 1.8^l	57.5 ± 1.88^f	55.5 ± 1.83^d	59.4 ± 1.74^h
	Broccoli	74.67 ± 5.66^j	66.9 ± 2.3^f	69.1 ± 2.3^h	62.5 ± 3.25^d
	Red young radish	91.93 ± 2.01^{jk}	89.03 ± 3.12^h	93.6 ± 4.11^l	90.33 ± 2.31^i
(B)					
Treatment	Sprout	Frequency			
		250 Hz	800 Hz	1 kHz	1.5 kHz
LT2	Alfalfa	68.57 ± 1.8^m	68.33 ± 1.75^m	63 ± 1.83^k	60.6 ± 1.85^i
	Broccoli	56.4 ± 3.44^a	56.9 ± 2.46^a	61.37 ± 1.75^c	60.4 ± 4.14^b
	Red young radish	80.83 ± 1.65^{bc}	81 ± 2.1^{bc}	81.07 ± 1.98^{bcd}	79.43 ± 2.12^a
LT3	Alfalfa	54.87 ± 1.81^c	53.57 ± 1.77^b	61.47 ± 1.86^j	59.33 ± 1.87^h
	Broccoli	64.33 ± 3.32^e	61.67 ± 3.46^c	75.37 ± 3.72^j	60.27 ± 2.89^b
	Red young radish	81.27 ± 2.35^{cd}	84.43 ± 2.54^f	81.63 ± 2.91^{de}	80.5 ± 2.84^b
LT4	Alfalfa	56.7 ± 1.93^e	51.93 ± 2.07^a	54.77 ± 1.87^c	60.53 ± 2.11^i
	Broccoli	64.3 ± 2.14^e	82.43 ± 3.31^k	68.33 ± 4.63^g	67.5 ± 1.99^f
	Red young radish	81.63 ± 3.61^{de}	81.63 ± 2.81^{de}	81.63 ± 3.46^{de}	81.93 ± 3.65^e

Table 3. FRAP analysis after short- and long-term sound wave treatment in alfalfa, broccoli and red young radish sprouts. (A) FRAP analysis after short-term sound wave treatment. (B) FRAP analysis after long-term sound wave treatment. The values represent mmol L^{-1} TE/100 mg FW. The untreated control values are 2.53 ± 0.03^d , 13.05 ± 0.03^h and 21.61 ± 0.3^{cde} for alfalfa, broccoli and red young radish, respectively. Each value represents the mean of three measurements ($\pm$ SE). Different letters indicate significantly different values ($P < 0.05$) calculated using ANOVA followed by a Duncan's multiple range test

(A)					
Treatment	Sprout	Frequency			
		250 Hz	800 Hz	1 kHz	1.5 kHz
ST2	Alfalfa	4.83 ± 0.07^j	3.01 ± 0.02^f	2.7 ± 0.04^e	3.18 ± 0.03^h
	Broccoli	14.37 ± 0.02^l	13.05 ± 0.04^h	11.08 ± 0.03^f	13.43 ± 0.02^j
	Red young radish	47.65 ± 0.58^j	36.06 ± 0.9^g	40.59 ± 2.67^h	36.1 ± 0.64^g
ST4	Alfalfa	5.52 ± 0.03^k	3.06 ± 0.05^{fg}	2.72 ± 0.05^e	3.14 ± 0.02^{gh}
	Broccoli	15.68 ± 0.05^m	13.47 ± 0.04^j	14.23 ± 0.03^k	9.87 ± 0.04^c
	Red young radish	45.41 ± 0.07^i	23.41 ± 2^e	55.82 ± 1.16^k	28.38 ± 1.59^f
(B)					
Treatment	Sprouts	Frequency			
		250 Hz	800 Hz	1 kHz	1.5 kHz
LT2	Alfalfa	6.49 ± 0.05^n	6.26 ± 0.03^m	5.67 ± 0.04^l	3.11 ± 0.05^{fgh}
	Broccoli	8.66 ± 0.05^a	8.69 ± 0.04^a	10.91 ± 0.02^e	10.08 ± 0.04^d
	Red young radish	19.3 ± 0.03^{abc}	17.47 ± 0.02^a	18.97 ± 0.04^{ab}	17.46 ± 0.03^a
LT3	Alfalfa	2.49 ± 0.01^d	2.23 ± 0.04^c	3.37 ± 0.06^i	3.09 ± 0.04^{fgh}
	Broccoli	13.41 ± 0.03^j	11.28 ± 0.02^g	16.43 ± 0.03^c	9.47 ± 0.04^b
	Red young radish	17.85 ± 0.03^{ab}	22.76 ± 0.14^e	19.57 ± 0.02^{abc}	18.2 ± 0.03^{ab}
LT4	Alfalfa	2.72 ± 0.04^e	1.23 ± 0.03^a	1.72 ± 0.02^b	3.12 ± 0.01^{fgh}
	Broccoli	13.01 ± 0.03^h	15.89 ± 0.05^n	13.01 ± 0.02^h	13.23 ± 0.03^i
	Red young radish	19.71 ± 0.21^{abc}	20.16 ± 0.06^{bcd}	21.51 ± 0.1^{cde}	22.22 ± 0.02^{de}

All FRAP values were higher in red young radish sprouts under ST treatment compared to the untreated control; these results are similar to the results for total flavonoid content and DPPH radical scavenging activity. The highest FRAP value (55.82 mmol L⁻¹ TE/100 mg) was detected after 1 kHz ST4 treatment, and the next highest was after 250 Hz ST2 and ST4 treatments (Table 3(A)). Under LT treatment conditions, most of the FRAP values were lower than those for the ST treatments, but under some conditions (800 Hz LT3, 1 kHz LT4 and 1.5 kHz LT4) values were similar to or slightly higher than that of untreated control (Table 3(B)). In summary, the DPPH radical scavenging activity and the FRAP value were slightly different, but the overall pattern of the DPPH and FRAP results was similar.

The different and diverse phenolic compounds present in plants could exhibit high FRAP reducing power; the greater the reducing power, the higher the electron donating ability.²⁷ Rice-Evans *et al.*²⁸ demonstrated that high levels of total phenolic compounds increase antioxidant activity. Indeed, in this study, the FRAP activity was high when total flavonoid contents were high as a result of sound wave treatment. These results indicate that in addition to total flavonoid content and DPPH radical scavenging ability, the antioxidant activity of FRAP increases in response to sound waves under specific conditions.

CONCLUSIONS

Here, we developed a sound wave-based technique to increase the total flavonoid levels in vegetable sprouts. We determined the best conditions (sound wave frequency, growth stage and total sound wave exposure time) for the production of high-quality vegetable sprouts (alfalfa, broccoli and red young radish sprouts) with increased total flavonoid content, which are easy to cultivate and of interest to consumers. Sound wave treatments affect the expression of genes influencing the production of various metabolites that induce various physiological changes in plants. So, further studies will be conducted for detailed mechanism identification. More systematic studies are needed to develop sound wave treatment technology that could be applied to other crops. Therefore, the results of this study provide the foundation for various investigations using sound waves in the future.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests in the research.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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