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Research Paper

Effects of Sound Stimulation on Physiological and Biochemical Responses of *Salvia splendens*

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Abstract

Plants are inevitably influenced by environmental stresses due to their immobility. Sound waves are one of these environmental factors affecting plants. The present study was designed to explore the less-known relationship between sound waves and plant responses. For this purpose, *Salvia splendens*, a common plant in urban green spaces, was exposed to sound waves at a frequency of 1000 Hz and at intensities of 90, 100 or 110 dB, as well as a control, for one hour a day for one month. Seedlings were obtained from the seeds of *Salvia*, were cultivated in an MS medium under *in-vitro* conditions and were sub-cultured every 20 days. The treatments were started 15 days after planting the seeds. The results of growth and antioxidant enzyme activities showed that the increase in the intensity of the sound waves at 1000 Hz frequency increased plant growth. Maximum root and shoot length, fresh weight and dry weight were observed at 110 dB. The sound waves also increased protein content and catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) enzymes activity. Malondialdehyde content was increased with an increase in intensity. Overall, *Salvia splendens* responded to the sound wave stress by improving growth, physiological and biochemical parameters.

Keywords: Environmental pollution, Floriculture, Landscape, Sound pollution, Stress.

INTRODUCTION

Salvia splendens is an herbaceous perennial plant belonging to the family Lamiaceae and is used as a garden plant in urban green spaces and rock gardens. It is also known for its remarkable resistance to pests, diseases, and drought (Clebsch and Barner, 2003). The relationship between stress and development is an important research field focused on by biologists and plant physiologists (Yiyao *et al*., 2000). An increase or decrease in any environmental factor may cause stress and provoke various plant responses. Scientists have shown that the development and other physiological processes of plant tissues and cells are greatly affected by stress (Zhao *et al.,* 2002; Hassanien *et al.,* 2014). Sound waves are one of the most important environmental stresses, which significantly affect plant growth and development and different plants have shown different responses to it (Yi *et al.,* 2003; Zhao *et al.,* 2002; Xiaocheng *et al.,* 2003; Qin *et al.,* 2003). Sound waves effectively increased free radicals in chrysanthemums and consequently their antioxidant enzymes (Xiujuan *et al.,* 2003).

Xiujuan *et al.* (2003) studied the effects of sound waves on antioxidant enzymes of chrysanthemums. The explants obtained from the tissue culture were exposed to sound waves with 1000 Hz frequency and 100 dB intensity at a rate of 1 hour/day for 0, 3, 6, 9,12 and 15 days. The results showed that MDA content and antioxidant enzyme activities were increased in the roots, stems, and leaves under the influence of the sound waves (Xiujuan *et al.,* 2003). A study on the effect of sound waves on *Dendrobium candidum* showed that the growth, enzyme activities, and MDA content were influenced by sound stimulations (Li *et al.,* 2008). Also, the effect of sound waves was observed on mung bean (*Vigna radiate*) growth and germination and kiwi fruit (*Actinidia chinensis*) callus proliferation (Cai *et al.,* 2014; Xiaocheng *et al.,* 2003). These studies confirmed the effect of sound waves on some plants and it can be concluded that sound waves affect plants, but they affect all plants in the same manner, whether perennial, seasonal or cut flowers. It is, however, essential to study the effects of sound waves on different plants in different conditions.

To clarify this issue, the present experiment aimed to study *Salvia splendens* as a perennial herbaceous plant exposed to sound wave stress at a certain frequency and different intensities.

MATERIALS AND METHODS

Plant materials

The seeds of *Salvia splendens* cv. 'Vista' were used for tissue culture to produce seedlings free of diseases and other environmental stresses. For disinfection, the seeds were submerged in running water for 5 minutes. Then, they were submerged in 70% ethanol for 5 minutes under a laminar flow hood in sterile conditions. Next, 10% sodium hypochlorite was applied to the seeds for 15 minutes. The seeds were rinsed by distilled water three times, each time for 5 minutes. A 1/2 MS medium without any growth regulator was used for the cultivation of seeds in which they were sub cultured once every 20 days. The plants were grown in an incubator set at 72 µmol m^{-2} s^{-1} light intensity, 16/8 day/night duration, and 70% relative humidity.

To apply the sound stress, an acoustic device was made in the Department of Electricity at Isfahan University. The desired frequency was generated by a function generator. The intensity was adjusted by an amplifier (Fig. 1). After 20 days of seed cultivation at the cotyledon stage, the treatments were started and applied at a rate of 1 hour/day for 30 days. The study was carried out as a completely randomized design with three replications (every experimental plot composed of three plants in one jam jar; Fig. 2). The sound treatments were applied according to Xiujuan *et al*. (2003) with the same frequency of 1000 Hz but different intensities of 90, 100 and 110 dB, as well as a control (no sound wave exposure).

Fig. 1. The acoustic device used to apply the sound waves to the plants.

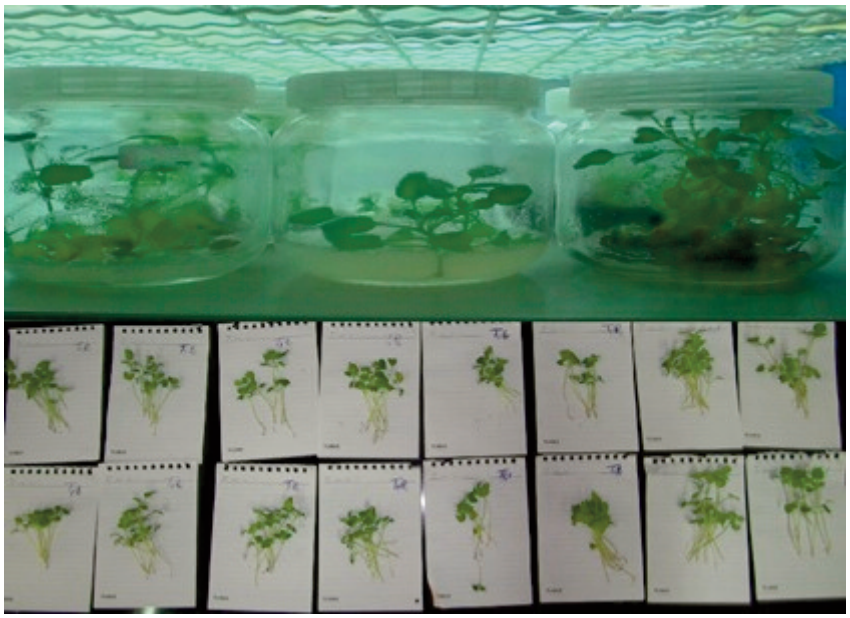

Fig. 2. The plants after the treatment and measurement.

Growth indices

Growth parameters such as stem and root length were measured with a ruler. Fresh weight was determined with a scale after the treatment and then the plants were dried in open air and shade for 30 days to find out their dry weight with a scale up to three decimal places.

Protein content

Protein content of the samples was extracted using the method developed by Cho and Seo (2005) with some modifications. For this purpose, 0.5 g of the frozen sample was poured into a mortar. Then, 2 mL of 100 mM phosphate buffer (extraction buffer) (pH 7.8) containing 0.1 mM EDTA and 1% polyvinylpyrolidone1 (PVPP) was added to the sample. The mixture was cen-

trifuged for 20 minutes at 12000 rpm at 4 ± 1°C. The obtained extract was used to measure protein content and activities of catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) enzymes.

Bradford's (1976) method was employed to measure protein content of the extract. For this purpose, 5 µL of the extracted protein was added and completely mixed with 295 µL Bradford solution for 15 minutes. Then, the absorbance of the mixture was recorded at 595 nm with a spectrophotometer (UV-160A; Shimatzo Corporation, Japan), and a BSA calibration curve was used to determine protein content in mg g^{-1} FW.

Enzyme activities

Catalase (CAT EC 1.11.1.6) activity was measured using a spectrophotometer with Aebi's (1984) method at 240 nm. For this purpose, 3000 µL potassium phosphate buffer (pH=7), 5 µL of 3.41 M hydrogen peroxide and 100 µL of enzyme extract were used. An enzymatic activity with a zero-second latency phase for 3 minutes was recorded at 20-second intervals.

The method developed by Mac Adam *et al.* (1992) was used to measure peroxidase (POD EC 1.11.1.7) activity. For this purpose, a spectrophotometer was used at 470 nm for 2 minutes at 30-second intervals. Three mL of sodium phosphate buffer with 50 µL of the extract was added to 3 µL of guaiacol and finally, 10 µL of hydrogen peroxidase was added to the sample. Three mL of sodium phosphate buffer, 3 µL of guaiacol, and 10 µL of hydrogen peroxide were used as a blank sample.

The method developed by Nakano and Asada (1981) was employed to measure ascorbate peroxidase (APX EC 1.11.1.11) activity with a spectrophotometer. The enzyme activity was measured using a 120-second delay phase in 180 seconds with 20-second intervals after adding the enzyme extract. Ascorbate peroxidase enzyme activity was determined based on the decomposition of H_2O_2 at 290 nm using a 2.8 mM cm^{-1} extinction coefficient.

Lipid peroxidation (MDA content)

Lipid peroxidation of the samples was determined by estimating malondialdehyde (MDA) content according to the method of Heath and Parker (1968). MDA extract was determined using 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The MDA content was calculated using the difference between spectrophotometric absorbance at 532 and 600 nm and 155 mM cm^{-1} extinction coefficient.

Statistical analysis

The experiment was carried out as a completely randomized design (CRD(with three replications and the data analysis was performed using SAS software (SAS Institute Inc., Cary, NC, USA v.9.4). The graphs were plotted using MS-Excel software. Means comparison was carried out by Duncan's test at the 1% probability level.

RESULTS

Growth measurements

The analysis of variance for the effect of the sound waves on stem length, root length, shoot weight, and shoot dry weight showed significant differences at the $P < 0.01$ level (Table 1).

The effect of the sound waves on growth indices of *Salvia splendens* is shown in Fig. 3. The impact of different intensities of sound waves was significant ($P < 0.01$) on stem length of *Salvia*. The highest stem length rate was observed in the 110 dB treatment and it increased by 69.5% compared to the control (Fig. 3A). Root length showed an ascending trend with an increase in intensity to 110 dB so that it was increased by 119% compared to the control (Fig. 3B). Shoot

weight showed an ascending growth as the intensity was increased although the difference was not significant between the control and 90 and 100 dB treatment (Fig. 3C). Shoot dry weight increased by 118% versus the control with an increase in the intensity of the sound waves to 110 dB (Fig. 3D).

Table 1. Analysis of variance for the effect of sound waves on physiological and biochemical indices of "*Salvia splendens*".

S.o.V	df	Stem length	Root length	Shoot weight	Shoot dry weight	Protein	CAT	POD	APX	MDA
Sound	3	6.075**	6.33**	0.00086**	0.000092**	3.36**	0.064**	0.34**	159.5**	232.7**
Error	12	1.16	2.11	0.000154	0.0000049	0.0098	0.0004	0.025	2.91	2.068
CV (%)		18.76	9.93	18.03	17.46	4.95	5.03	10.97	2.07	5.22

*, ** and ^{ns}: Significant at $P < 0.05$, $P < 0.01$ and insignificant, respectively.

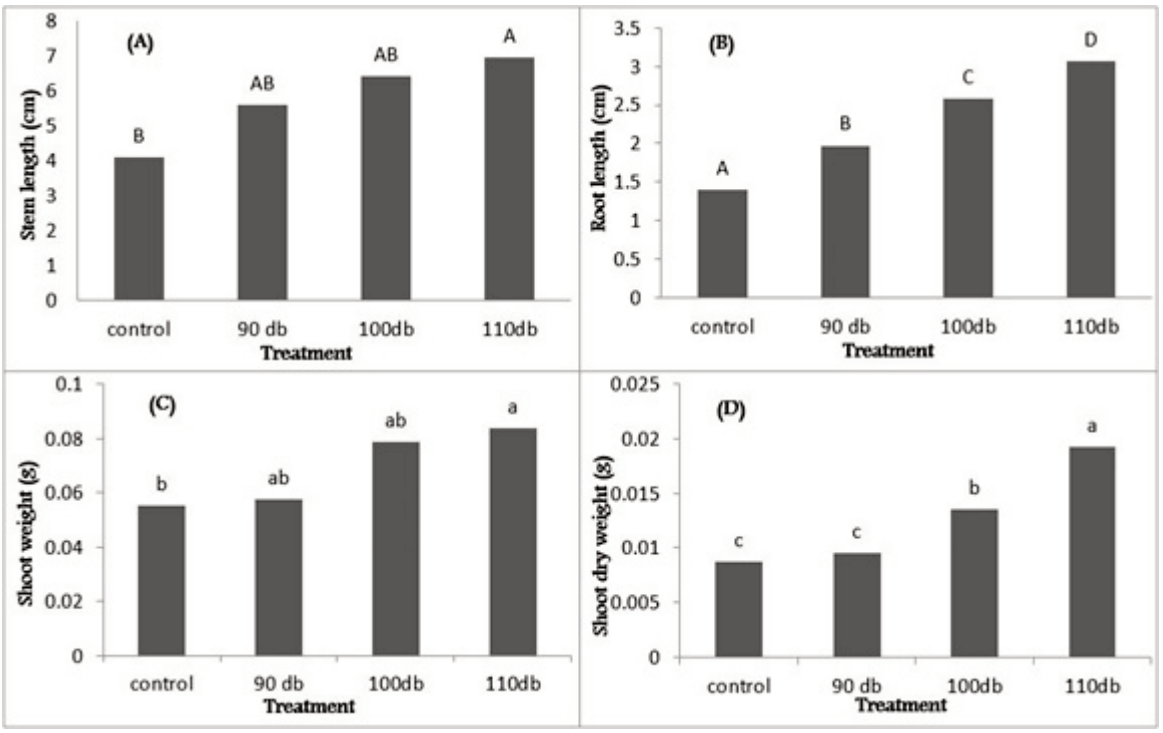

Fig. 3. The effect of sound waves on stem length (A), root length (B), shoot weight (C), and shoot dry weight (D) of *Salvia splendens.* Values with the different letter(s) are significantly different according to Duncan's multiple range test at P<0.01.

Biochemical indices

Based on the results in Table 1, there was a significant difference in protein content, catalase, peroxidase, and ascorbate peroxidase enzyme activities at $P < 0.01$ under different intensities of the sound waves (Table 1).

According to Fig. 4A, with an increase in the sound wave intensity, the protein content of the plants was increased and the highest protein content was observed in the plants exposed to 110

dB intensity (3.1 mg g^{-1} FW). According to Fig. 4B, the CAT enzyme activity initially showed a downward trend followed by an upward trend. Low enzyme activity was observed at 90 dB (0.33 µmol g^{-1} min^{-1} FW) and the maximum enzyme activity was observed at 110 dB (0.62 µmol g^{-1} min^{-1} FW). As shown in Fig. 4C, the POD enzyme activity in *Salvia splendens* was increased under the influence of the sound waves with an increase in the intensity and the maximum POD activity was observed at 110 dB (1.84 µmol g^{-1} min^{-1} FW). According to Fig. 4D, the APX enzyme activity declined at first (76 µmol g^{-1} min^{-1} FW), but the maximum enzyme activity was observed at 110 dB (89.29 µmol g^{-1} min^{-1} FW).

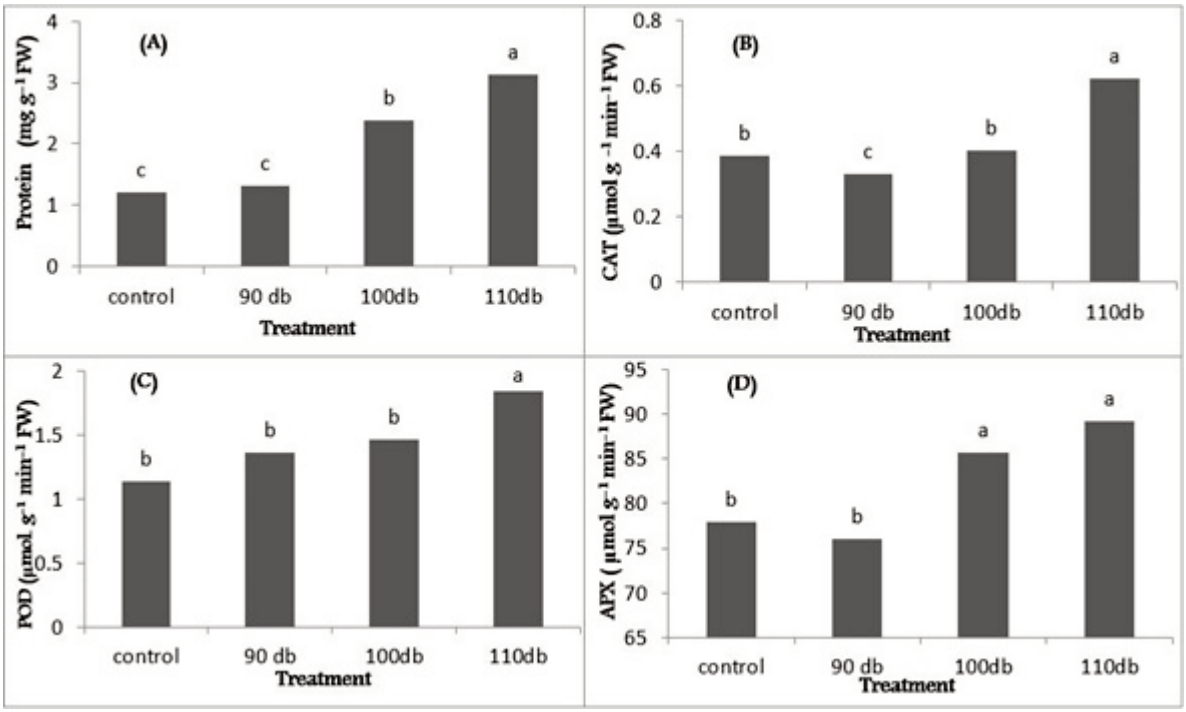

Fig. 4. The effect of sound waves on protein content (A), CAT (B), POD (C), and SOD (D) of *Salvia splendens.* Values with a different letter are significantly different according to Duncan's multiple range test at P<0.01.

According to the results, it can be concluded that the sound waves had a significant effect on the MDA content at P < 0.01 (Table 1). The effect of different intensities of the sound waves on the MDA content as an indicator of cellular damage is shown in Fig. 5. The MDA content was gradually increased as the intensity was increased. The MDA content was maximized at the intensity of 110 dB (37.17 µmol g^{-1} FW).

DISCUSSION

Environmental factors greatly affect biochemical and physiological traits in plants. These traits profoundly influence growth, survival, proliferation, and aging in plants (Sharma *et al.,* 2012). Sound waves as intermittent stress may have a great impact on plant growth. Many scientists have proven that low-frequency and low-intensity sound waves do not impair cellular structure and even increase enzyme activity, membrane permeability, and RNA synthesis (Xiujuan *et al.,* 2003).

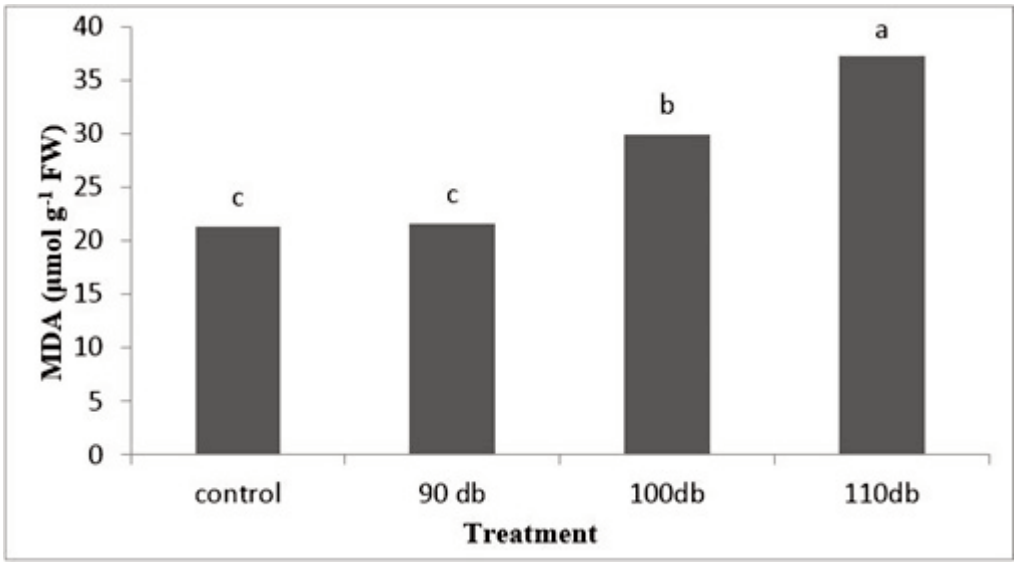

Fig. 5. The effect of sound waves on MDA content of *Salvia splendens.* Values with a different letter are significantly different according to Duncan's multiple range test at $P<0.01$.

Growth indices, such as shoot fresh and dry weight, shoot length, and root length, were measured under different intensities of sound waves at 1000 Hz frequency. The results revealed that the plant growth indices were increased as the intensity of sound waves was increased. However, no significant difference was found at the intensity of 90 dB among the growth indices. Environmental stresses usually reduce plant growth due to increase from normal range (Heidari *et al.,* 2019). However, some weak environmental stresses improve plant tolerance and effectively increase plant growth (Yi *et al.,* 2003). Bochu *et al.* (1998) investigated the effect of sound waves on plants for the first time. They reported that the sound waves with a certain frequency, intensity, and treatment period not only had no destructive effects on plants but they also improved cell growth and cell division in carrots. Physiological reasons, such as increased absorption and metabolism of plants, may lead to plant growth under the influence of sound waves. The same case scenario was observed in two species of Chinese cabbages and cucumbers at two stages of growth under the influence of sound waves (Qin *et al.,* 2003). In addition, the effect of sound waves on the metabolism of kiwi fruits was studied. The results showed that the sound waves with a certain frequency and intensity increased the metabolism of the plants. Furthermore, ATP content was increased under the influence of sound waves with 1000 Hz frequency and 100 dB intensity (Xiaocheng *et al.,* 2003). In addition to physiological factors, hormones may also increase plant growth under the influence of sound waves. The effect of sound waves on callus growth was studied in chrysanthemums. Callus growth was increased at 800 Hz frequency and 100 dB intensity. In this period, the IAA oxidase activity was decreased, and this increased auxin hormone that stimulated callus growth (Yiyao *et al.,* 2002).

Soluble protein content was increased with the intensity of the sound waves. This is shown in Fig. 5 for *Salvia splendens*. Increased soluble protein content under the influence of sound waves has also been reported in chrysanthemums and *Nicotiana tabacum*. The reasons for this increment lie in the fact that the protein content of cellular membrane is increased in addition to the non-structural protein content such as enzymes. Increased protein content under the influence of different intensities and frequencies of sound waves were consistent with the results of Zhao *et al.* (2002). Therefore, physiological, hormonal and genetic factors are involved in changing plant

growth and protein content under the influence of sound waves.

Antioxidant enzymes are cited as other parameters that change physiological and growth indices in plants under the influence of sound waves (Chowdhury *et al.,* 2014). Oxidative damage is an effective barrier to plant growth and proliferation in the absence of proper environmental conditions. Most damage to plants by various environmental stresses is due to oxidative damage at different cellular levels. Plants possess a highly efficient protective system in response to oxidative stress. This protective system destructs or neutralizes free radicals and contains such enzymes as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), peroxidase (POD), and glutathione reductase (GPX) (Sharma *et al.,* 2012). The effect of sound waves on antioxidative enzymes was studied in chrysanthemums. The sound waves at 1000 Hz frequency and 100 dB intensity and different treatment periods increased the production of CAT, POD, and SOD. The CAT and POD enzymes scavenge hydrogen peroxide (H_2O_2) and superoxide dismutase scavenge O_2 (Xiujuan *et al.,* 2003). CAT is an iron-containing protein and antioxidant enzyme, which is activated as H_2O_2 increases in the cells of plants and animals (Mittler, 2002). The production of CAT was increased under the influence of 1000 Hz and 90, 100, 110 dB intensities of the sound waves in the shoots of *Salvia*. POD is one of the antioxidant enzymes in plant and animal cells. This enzyme effectively increases resistance to stress caused by reactive oxygen species (ROS) in plants. POD converts H_2O_2 into water (H_2O) and oxygen (O_2) (Sharma *et al.,* 2012). Ascorbate peroxidase (APX) is another antioxidant enzyme found at high concentrations in chloroplasts, cytosol, vacuole, and apoplast in leaf mesophyll. Scholars suggest that APX is the most important antioxidant enzyme in plants and acts as a regenerative agent for many free radicals, especially H_2O_2 (Mittler, 2002). APX was increased in the plants exposed to the sound waves as shown in Fig. 4D. Although APX was decreased at the intensity of 90dB, this decline occurred due to the enzyme recovery period. The APX enzyme activity was increased at the intensities of 100 dB and 110 dB, which showed an increase in plant resistance against oxidative stress. The effect of thermal stress on *Salvia* cv. 'Vista' and 'King' at different periods in terms of the CAT, POD and SOD enzymes showed that 'Vista' was a heat-tolerant variety. ROS's were produced and antioxidant enzymes were increased in 'Vista' in thermal stress. Antioxidant enzymes showed a downward trend in thermal stress in 'King', which was a heat-susceptible variety. These results about 'Vista' are consistent with those obtained in the present study (Liu *et al.,* 2013).

ROS plays an important role in cellular aging. Membrane lipid peroxidation is one of the main features involved in cellular aging. Lipid peroxidation is intensified by ROS and lipoxygenase enzyme. It should be noted that biological and abiotic stress stimuli increase the synthesis of ROS in plant cells. Oxidative damage is caused by separation of cellular membrane lipids, release of fatty acids, which provide the substrate for lipoxygenase enzymes that cause membrane lipid peroxidation and exacerbate cellular aging in plant (Sharma *et al.,* 2012).

MDA content as an indicator of cellular membrane damage due to oxidative stress was measured in *Salvia*. As shown in Fig. 5, no significant difference was observed in MDA content at 90 dB intensity while MDA content showed an upward trend at 100 dB and 110 dB intensities. This reflects the gradual destruction of cellular membrane lipids by ROS. These results are consistent with those obtained by other scholars for the effect of sound waves. MDA content was measured under the influence of sound waves at 1000 Hz frequency and 100 dB intensity in chrysanthemums. MDA content was increased in this plant as the number of days of exposure to sound waves was increased. The maximum MDA content was measured in shoots (Li *et al.,* 2008). MDA as a biomarker of cellular membrane destruction was measured in *Salvia* 'King' and 'Vista' under thermal stress at different periods. A significant difference was found and MDA content showed an upward trend by the increase in thermal stress. These results are consistent with those obtained in the present study in terms of sound waves (Liu *et al.,* 2013).

CONCLUSION

The findings showed that the increase in MDA content and antioxidant enzyme activities along with the increase in growth of *Salvia* resulted in the rapid vegetative growth to complete growth stages prior to plant death under sound wave stress. On the other hand, plant growth increased plant resistance against sound wave stress. Vegetative growth is accelerated to allow the initiation of the flowering phase and ensure plant survival. The scientific principles of the study could be strengthened with further long-term studies where *in vitro* conditions are replaced with plantation in the field, the reproductive phase can be observed and approximate time of transition from the stage of juvenility to flowering can be determined.

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