

Changes in antioxidant enzymes activity and physiological traits by exogenous salicylic acid in basil (*Ocimum basilicum*) under Pb stress

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Abstract

Salicylic acid (SA) is a key molecule that alleviates abiotic stress in many plant species. In this study, the role of SA was examined to moderate lead (Pb) toxicity in the basil (*Ocimum basilicum*). The experimental design was a randomized complete block design with 4 levels of PbNO₃; 0 (control), 100, 200 and 300 mg kg⁻¹ of soil as the first factor, and the foliar application of SA at 3 levels of 0, 50 and 100 mL L⁻¹ comprised second factor that were applied with four repetitions. The experiment was conducted during 2013 at research greenhouse of the Zabol University. Addition of Pb at a rate of 300 mg.kg⁻¹ significantly reduced the carotenoids by 45.5 percent and organic acids by 49.3 percent, while it caused an increase in anthocyanins, flavonoids, electrolyte leakage and antioxidant enzymes, including lipoxygenase and glutathione peroxidase. In addition, SA spraying had a significant influence on all traits. In this study, the interaction effects between SA and Pb were significant on carotenoids, anthocyanins, flavonoids, electrolyte leakage, organic acid, and lipoxygenase and glutathione peroxidase, and plaid a moderating role and reduced the harmful effects of Pb toxicity. The results of this experiment suggested that the application of SA in basil caused a reduction in oxidative damage originated by Pb toxicity and induce the synthesis of photosynthetic pigments, such as carotenoids.

Keywords: Abiotic stress, Flavonoids, Heavy metals, Pot experiment, Stress mitigator

Introduction

The contamination of soil by heavy metals is one of the main environmental problems in human societies as a result of industrial and agricultural activities. Among the heavy metals, lead (Pb) is one of the toxic heavy metals with unknown biological effects that easily accumulate in sediments and soils (Mishra and Choudhuri, 1999). One of the most notable effects of the Pb is the induction of oxidative stress in plants and the formation of reactive oxygen radicals. The different types of Reactive Oxygen Species (ROS) as the main causes of oxidative stress have the high oxidizing capacity, threaten bioluminescence and different cellular processes, interrupt normal metabolism and ultimately lead to cell death (Panda *et al.*, 2003). Antioxidant systems are considered a tolerance mechanism to reduce the toxicity of heavy metals in plants. These antioxidant systems include enzymes such as catalase, glutathione reductase, glutathione peroxidase, superoxide dismutase and the family of peroxidases and non-enzymatic antioxidants, such as ascorbates, glutathione, flavonoids, α -Tocopherol and carotenoids (Apel and

Hirt, 2004). Dat *et al.* (2000) and Prasad. (2003) reported an increase in the synthesis of glutathione peroxidase and catalase enzymes under stress conditions. These protective mechanisms, such as carotenoids, flavonoids, anthocyanins and ascorbic acid, protect the plant against oxidative stress by sweeping free radicals (Woodson and Lawton, 1988). Carotenoids generally play a role in the deactivation of single oxygen and in the scavenging of free radicals, which is due to their ability to transfer energy to photosynthesis and the role of optical protection (Jithesh *et al.*, 2006). Flavonoids have hydroxyl and carboxyl groups that can be specifically bound to the elements (Jung *et al.*, 2003). The results of the treatment with heavy elements in common beans (*Phaseolus vulgaris*) showed a significant increase in flavonoids (Sakihama and Yamasaki, 2002). In general, it is believed that flavonoids prevent oxidative damage by sweeping several reactive oxygen species and disrupting radical chain reactions during lipid peroxidation.

The role of salicylic acid (SA) as a key molecule in the alleviates abiotic stress has already been well

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described. The application of exogenous SA could provide protection against various types of stresses such as heavy metals (Belkhadi *et al.*, 2010). The literature shows that the treatment of plants with low concentrations of SA could have an effect similar to that of acclimatization, causing a greater tolerance to most types of abiotic stress due mainly to the increase in capacity of antioxidative. Although SA can also cause oxidative stress in plants, partially through the accumulation of hydrogen peroxide (Mishra and Choudhuri 1999; Ghai *et al.*, 2002). The effect of exogenous SA depends on many factors, such as the mode of application, the endogenous level of the SA and its concentration in the given plant, the stage of development and the species of the plant. Recent research has indicated that not only the application of exogenous SA alleviates the effects of stress, but that abiotic stress could also change the levels of endogenous SA in the tissues of plants (Belkhadi *et al.*, 2010).

Basil (*Ocimum basilicum* L.) is used as a medicinal plant, spices and fresh herbs. The aromatic leaves of this plant are applied as flavor of food, sweets and drinks. The ingredient of this plant is appetizing and is used to treat bloating and gastrointestinal augmentation (Marrotti *et al.*, 1996).

In the present study, due to the antioxidant properties and the pseudo-hormonal role of SA, the role of this compound in controlling the negative effects of Pb on basil plant was investigated.

Materials and methods

The experiment was conducted to investigate the effects of salicylic acid (SA) spraying on basil (*Ocimum basilicum*) antioxidant defense system characteristics under Pb stress in Research Greenhouse of Zabol University during the autumn and winter of 2013.

The experimental design was a factorial experiment in a randomized complete block design with three replications. Application of Pb as lead nitrate ($\text{Pb}(\text{NO}_3)_2$) in four levels included 0 (control), 100, 200 and 300 mg of Pb kg^{-1} of soil as first treatment and SA spraying in three levels (0 (deionized water spraying), 50 and 100 mL L^{-1} of tap water) comprising second treatment. Lead nitrate and SA (MERK, Germany) provided by the Research Laboratory, Faculty of Agriculture, University of Zabol. In this study, the sweet basil seeds of Keshkeni Luvelou were used. The physical and chemical properties of the soil before the experiment are presented in Table 1.

To determine the chemical properties of soil, samples were taken from the sieve after air drying.

Before sowing, all the pots received 200 mg of nitrogen per kg of soil (in the form of urea), 100 mg of phosphorus per kg of soil (in the form of super phosphate) and 100 mg of potassium per kg of soil (in the form of potassium sulphate). Different amounts of Pb based on the same amount of soil for each pot (approximately 2 kg pot^{-1}) were calculated and mixed

with the soil. The mixed soil was kept in plastic bags for three weeks (Ratushnyak *et al.*, 2012) in a room at 25-30°C and 60-70% RH and then the pots were filled with this soil and the five germinated seeds were planted at a depth of 1 cm on March 27. After 2 weeks, six germinated plants were kept at a distance of 2 cm between the plants. In the stage of 6 leaves (approximately 30 days after germination) SA spraying was applied to the plant. The pots were watered every two days. The plants were harvested in a vegetative state ten weeks after sowing. The evaluated properties in this study included anthocyanins, flavonoids, electrolytescarotenoids and organic acids, as well as the activity of antioxidant enzymes of glutathione peroxidase (GPX) and lipoxygenase (LOX) in the aerial parts of the plant.

The carotenoid content of the samples was measured at 470 nm by the method of Arnon (Arnon, 1967) using a spectrophotometer (Unico UV-2100- USA). The anthocyanins were measured according to Wagner *et al.* (1979) at 550 nm and reported on the basis of mM g^{-1} of fresh weight. The flavonoids were determined using the method Krizek (Krizek *et al.*, 1998) at wavelengths of 270, 300 and 330 nm. The measurements of electrolyte leakage were carried out using the method of Ben-Hamed *et al.* (2007). The measurements of organic acids were carried out in accordance with Krizek *et al.* (1998). In addition, the measurements of glutathione peroxidase were carried out by the method of Pagiha (Pagiha and Valentine, 1987) and lipoxygenase by the method of Doderer *et al.* (Doderer *et al.*, 1992) at wavelengths of 340 and 234 nm, respectively, and were presented based on mM mg^{-1} protein.

The analysis of the data was performed with SAS 9.1 software and the average of the data was compared with LSD test at 5% level.

Results

Anthocyanin: The interaction between Pb and SA at different levels of stress (100, 200 and 300 mg kg^{-1} of soil) was significant on anthocyanin ($P \leq 0.01$). For example, in the fourth level of pb (300 mg kg^{-1} of soil), with an increase in SA (50 mL L^{-1}), anthocyanin decreased by 10.2% compared with the control (without stress) (Figure 1a).

Carotenoid: The effect of stress of Pb on carotenoid content was significant at 1% probability level (Table 2). With increasing Pb level, carotenoid levels decreased. The highest amount of carotenoids corresponded to the control (without stress) with an average of 24.46 mg g^{-1} of fresh weight and the lowest was observed in the fourth level of Pb (300 mg kg^{-1} of soil) with an average of 11.45 mg g^{-1} of fresh weight, and the application of SA at a level of 50 mL L^{-1} resulted in a 12% increase in carotenoid content compared to the control (without stress) (Table 3).

The interaction of pb and SA had a significant effect on the carotenoids ($P \leq 0.01$). For example, at the third level of Pb (200 mg kg^{-1} of soil), with the increase of

Table 1. Physical and chemical properties of the soil

EC	pH	N	P	K	Mn	Fe	Zn	Clay	Silt	Sand	Soil texture
(dSm ⁻¹)		(%)			(mg kg ⁻¹)				(%)		
1.8	7.1	0.06	12	185	3.1	2.2	4.8	32	27	41	Clay loam

Table 2. Analysis of variance for anthocyanins, carotenoids, and flavonoids in basil influenced by lead (Pb) toxicity and salicylic acid (SA) spraying of basil

SOV	df	Anthocyanin	Carotenoid	Flavonoids 270 nm	Flavonoids 300 nm	Flavonoids 330 nm
Replication	2	0.0025**	0.022**	0.00052**	0.00213**	0.00706**
Pb	3	0.4269**	12.351**	0.23243**	0.31585**	0.23082**
SA	2	0.0110**	2.980**	0.00384**	0.00510**	0.00901**
Pb×SA	6	0.0011**	0.173**	0.00028**	0.00114**	0.00109 ^{ns}
Error	22	0.000065	0.044	0.000028	0.000605	0.000470
CV (%)	-	1.77	7.25	0.77	5.24	3.85

ns, ** and * are non-significant, significant at the 5 and 1% probability levels, respectively

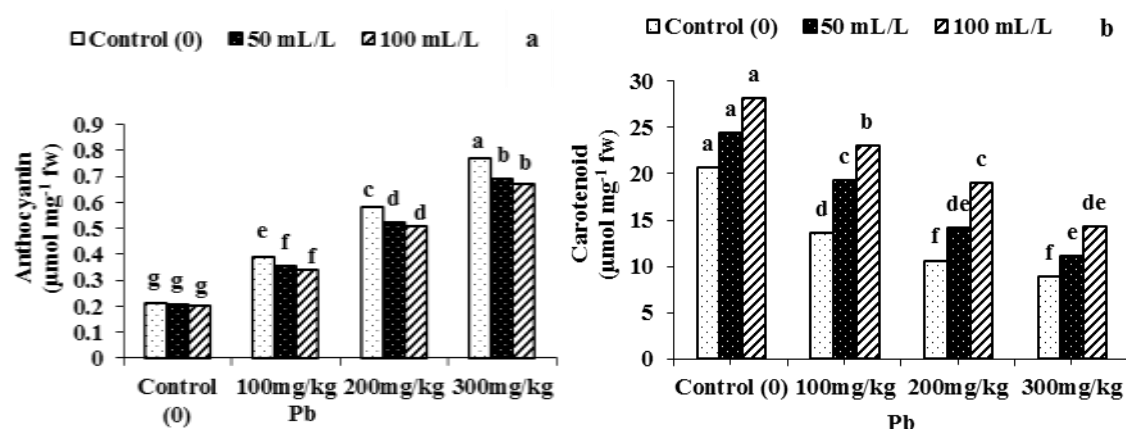


Fig. 1. Interaction effects of lead (Pb) toxicity and salicylic acid (SA) spraying on the concentration of anthocyanin (a) and carotenoid (b) of basil aerial parts. Similar letter represents not significantly different at the 0.05 level probability by LSD Test. Results are an average of three repetitions.

Table 3. Analysis of variance for organic acids, electrolyte leakage content and glutathione peroxidase and lipoxigenase enzyme activity in basil influenced by lead (Pb) toxicity and salicylic acid (SA) spraying of basil

SOV	df	Organic acids	Electrolyte leakage	Glutathione peroxidase	Lipoxigenase
Replication	2	0.0021*	0.00003 ^{ns}	0.000006**	0.0003**
Pb	3	0.0310**	0.47021**	0.000846**	0.2919**
SA	2	0.0039**	0.00844**	0.000075**	0.0077**
Pb×SA	6	0.0003**	0.00104**	0.000011**	0.0006**
Error	22	0.000088	0.000039	0.0000005	0.0000015
CV (%)	-	4.37	0.99	3.35	0.26

ns, ** and *: are non-significant, significant at the 5 and 1% probability levels, respectively.

SA (50 mL L⁻¹), the level of carotenoids increased by 15.3% compared to the control (Figure. 1B).

Flavonoids: The effect of stress of Pb and SA on the flavonoid content at wavelengths of 270, 300 and 330 nm was significant at a 1% probability level (Table 2). As the Pb stress increased, the content of flavonoids increased in the aerial parts of the basil, however, when increasing the SA (level of 100 mL L⁻¹), flavonoid kinds (270, 300 and 330 nm) were reduced by 1.5%, 3.8% and

9.1%, respectively, compared to the control (without stress) (Table 3).

The interaction of Pb and SA on flavonoids (270 nm) was significant ($P \leq 0.01$), for example, in the fourth level of Pb (300 mg kg⁻¹ of soil), the increase in SA (100 mL L⁻¹) decreased the flavonoids (270 nm) and indicated an increase of 1.6% compared to the control. In addition, the interaction of Pb and SA reduced the flavonoids detected at 300 and 330 nm, but this decrease

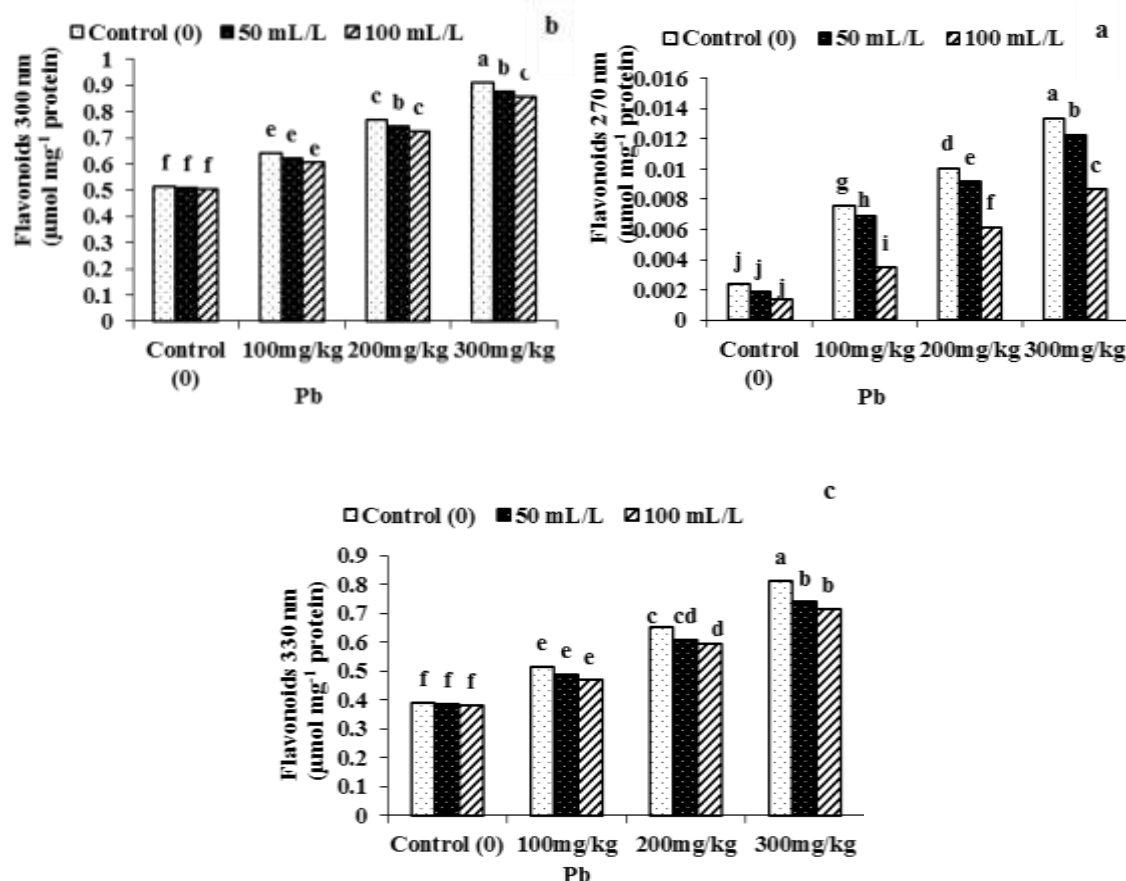


Fig. 2. effects of the interaction of the toxicity of lead (Pb) and salicylic acid (SA) on the concentration of flavonoid 270 nm (to), flavonoid 300 nm (b) and flavonoid 330 nm (c) of the aerial parts of basil. A similar letter does not represent a significant difference in the probability level of 0.05 according to the LSD Test. The results are an average of three replicates

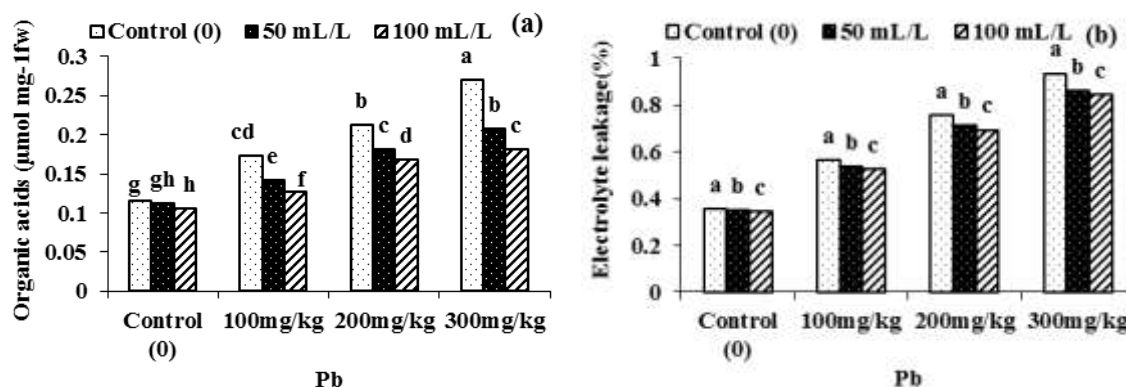


Fig. 3. Effects of the interaction of the toxicity of lead (Pb) and salicylic acid (SA) on the concentration of organic acids, leaks of electrolytes in the aerial parts of the basil. A similar letter does not represent a significant difference in the probability level of 0.05 according to the LSD Test. The results are an average of three replicates

was not statistically significant (Figure. 2a,b,c).

Organic acids: The results indicated that the treatment with Pb at a 1% probability level had a significant effect on the content of organic acids. The greatest reduction in organic acids was related to the fourth level of Pb (300 mg kg⁻¹ of soil) with an average of 141% and the lowest decrease was related to non-stress (control) with an average of 0.278% (Table 2). The interaction between Pb and SA at different levels of

Pb (100, 200 and 300 mg kg⁻¹ of soil) was significant on organic acid ($P \leq 0.01$). For example, at the third level of Pb (100 mg kg⁻¹ of soil), with the application of SA at a rate of 50 mL L⁻¹, organic acid increased by 17.1% compared to the control (non-spraying) (Figure. 3a).

Electrolyte leakage: The effect of Pb and SA was significant at a 1% probability level on the electrolyte leakage in the basil plant (Table 2), so that the second level of Pb (100 mg kg⁻¹ of soil) increased 35.6%

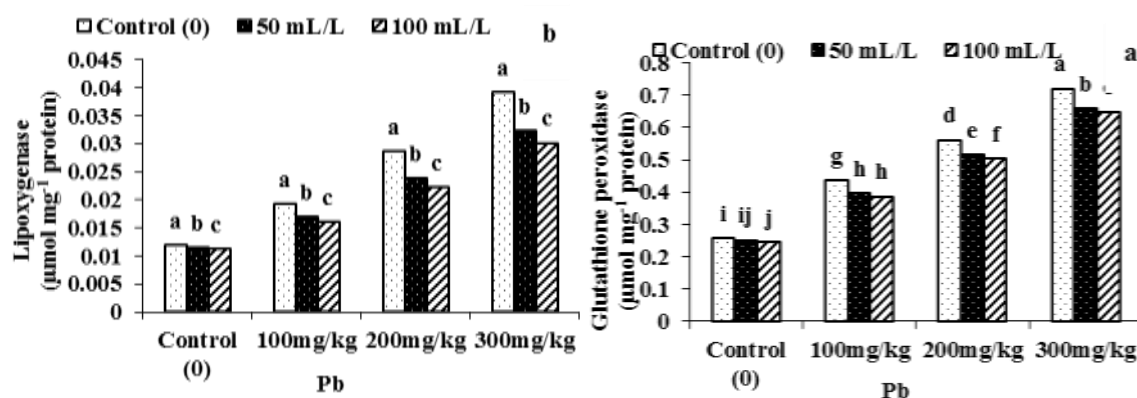


Fig. 4. Interaction effects of lead (Pb) toxicity and salicylic acid (SA) spraying on the activity of glutathione peroxidase (a) and lipoxygenase of basil aerial parts (b). Similar letter represents not significantly different at the 0.05 level probability by LSD Test. Results are an average of three repetitions.

electrolyte leakage compared to the first level (without stress), and the use of SA (level 100 mL L⁻¹) caused a 7.7% reduction in electrolyte leakage compared to the control.

The interaction of stress of Pb and spraying of SA at different levels of Pb (100, 200 and 300 mg kg⁻¹ of soil) was significant on electrolyte leakage ($P \leq 0.01$). For example, in the second level of Pb (100 mg kg⁻¹ of soil), with increasing SA (100 mL L⁻¹), the electrolytic leakage reduced by 6.7% compared to the control (without stress) (Figure 3a).

Glutathione Peroxidase (GPOX) and Lipoxygenase (LOX)

The effect of treatment with Pb and SA spraying was significant on the activity of lipoxygenase and glutathione peroxidase at a probability level of 1% (Table 3). The interaction of SA and Pb at different levels of Pb (100, 200 and 300 mg kg⁻¹ of soil) was significant on the activity of glutathione peroxidase and lipoxygenase ($P \leq 0.01$) and in the fourth level of Pb (300 mg kg⁻¹ of soil), the SA at the level of 100 mL L⁻¹ increased the activity of glutathione peroxidase and lipoxygenase, respectively, 0.30 and 0.160 μmol mg⁻¹ and of protein, respectively (Figure. 4a and b).

Discussion

Biotic and abiotic stress caused a series of reactions in the plants. One of the environmental stresses is the toxic metals in the soil that interrupt the life cycle and activate several biochemical reactions (Hamid *et al.*, 2010). Pb is one of the toxic metals for humans that does not have a known biological function; however, due to the relatively high solubility of Pb in water, it is easily absorbed by the root system. SA (2-hydroxy benzoic acid) is a phenolic compound and one of the plant hormones that exists in all organs of the plant. The concentration of this hormone increases when the plant is exposed to biotic and abiotic stress (Kranterev *et al.*, 2008). According to Amo Aghaei *et al.* (2012), the increase in cadmium concentration reduced the amount of carotenoids by 41.3% and increased the ion

electrolyte leakage rate by 58.1%. In addition to, SA pretreatment of plants exposed to cadmium toxicity caused a 29.5% increase in carotenoids and a 29.7% reduction in electrolyte leakage and thereby reducing cadmium toxicity. These findings are in line with our results and also the findings of El-Tayeb (2005) in sunflower plants grown under copper toxicity. In addition, Popova *et al.* (2005) have reported that the electrolyte leakage rate was higher in the peas treated with cadmium than in the control plants. Haghighi *et al.* (2010) in their research on lettuce expressed an addition of 2 and 3 mg of cadmium kg⁻¹ of soil, organic acids decreased by 18 and 30%, respectively compared to the untreated plants.

To cope with oxidative stress induced by Pb, plants use different defense mechanisms called enzymatic and non-enzymatic antioxidants. Increasing the metabolism of flavonoids and the number of phenolic compounds can be observed under various environmental factors and stress conditions. An increase in soluble flavonoids, such as those involved in lignin biosynthesis, can cause changes in anatomy during stress (Michalak, 2006). Flavonoids also increase the stability of the cell wall and create a physical barrier to protect cells against the damaging effects of heavy metals (Diaz *et al.*, 2001). Phenolic compounds, which include lignins, phenolic acids, coumarins, anthocyanins, and flavonoids, are synthesized from phenylpropanoid metabolism pathway. These secondary metabolites can act as antioxidants, free radical extinguisher or sweepers in plants (Solecka, 1997). The increase in anthocyanins is probably due to the increased activity of the PAL enzyme (Chen *et al.*, 2006). Flavonoids and anthocyanins in the leaf act as free radical receptors and protect plants from oxidative stress (Christoffersen and Laties, 1982).

In this study, an increase in electrolyte leakage was observed in all Pb treatments and the highest electrolyte leakage was obtained in situations of severe stress and the lowest electrolyte leakage in the untreated control plants. In addition, the lowest electrolyte leakage was

observed in non-treated plants with salicylic acid, and the use of salicylic acid could improve the amount of this trait. The electrolyte leakage shows the amount of damage to the cell membrane. Non-living stress can increase the permeability of bio-membranes by damaging the cell and, therefore, increase electrolyte leakage and inactivate membrane proteins, which in turn reduce the activity of the membrane, and decrease the ability of the cell membrane to obtain water and soluble materials and, consequently, lead to cell death (Jabbarzadeh *et al.*, 2009).

By reducing the amount of free radicals, acid salicylic acid reduces lipid peroxidation and ion leakage of from the membrane, which protects the plants from the stress of heavy metals (Senaratna *et al.*, 2003; El-Tayeb, 2005). It has also been reported that salicylic acid protects the membrane through the action of organic acids such as putrescine, spermin and spermidine, as well as the formation of stable membrane complexes (Nemeth *et al.*, 2002). Bandurska and Stroinski (2005) have reported that damage caused by water deficiency in the leaf cell membrane decreased in the treated barley plant before treatment with salicylic acid. The reduction of damage to the membranes due to the use of salicylic acid, known as a way to increase resistance to stress in plants, may be associated with the production of antioxidants to reduce damage by oxidation. It has been reported that salicylic acid significantly reduces ionic leakage and accumulates toxic ions in the plant and also increases the cytokinins (Kranterev *et al.*, 2008).

Pour Mousavi *et al.* reported that under conditions of severe stress, electrolyte leakage was greater than that of mild stress and non-stress. Mehrabian Moghadam *et al.* (2011) the studies showed that salicylic acid under conditions of drought stress, it reduced the amount of electrolyte leakage. Najafian *et al.* (2009) stated that high concentrations of salicylic acid (450 ppm) under salinity conditions increased the amount of electrolyte leakage in thyme. Similarly, the highest concentrations of salicylic acid used in this study have been effective in improving membrane stability and have not shown any toxicity. Stevens *et al.* (2006) have stated that the concentration of 0.1 mM SA reduced the amount of tomato electrolyte leakage at a concentration of 150 mM NaCl by 44% and in a concentration of 200 mM up to 32%.

Rab and Saltveit (1996) have stated that stress increases electrolyte leakage and increases the concentration of harmful oxygen compounds. The accumulation of these toxic compounds can lead to lipid peroxidation of the cell membrane and organelles, which ultimately causes physiological disorders and the appearance of cold stress in plants (Takac, 2004). In an experiment with corn seedlings, the amount of electrolyte leakage increased under cold stress conditions (Ali *et al.*, 2010).

Mazaheri Tirani *et al.* (2008) have concluded that by increasing the level of 0.5 and 1 mM of SA through the accumulation of soluble substances (ascorbate and dihydroascorbic acid), anthocyanin and flavonoids protect macromolecular molecules and cell membranes and reduce the destructive effects of ethylene in the plants.

Glutathione peroxidase is one of the enzymes that plays an important role in the management of environmental stress. This enzyme catalyzes the reduction of hydrogen peroxide using resuspended glutathione and protects cells against oxidative damage (Dixon *et al.*, 1998). It has been shown that the increase in lipoxygenase activity under stress conditions leads to the oxidation of fatty acids bound to the membrane and the release of lipid peroxidation (Molassiotis *et al.*, 2006). Kazemi *et al.* (2010) when studying the effect of SA and nickel stress on the activity of lipoxygenase enzyme concluded that, as stress increased, the activity of this enzyme in both levels of nickel treatment increased. However, SA has modified the activity of this enzyme in stress treatments. It has also been reported that the cause of growth stimulation by SA may be related to an increase in antioxidants in the cell, which protects plants against the oxidative degradation of heavy metals (El-Tayeb, 2005).

Conclusion

In general, it is concluded that the effect of stress caused by the increase of the concentration of Pb in the physiology of the plants is different. Reductions in organic acid, carotenoid pigmentation and increased electrolyte leakage and enzymatic and non-enzymatic antioxidants such as anthocyanins, flavonoids, and lipoxygenase enzymes and glutathione peroxidase in the leaves indicate the effects of toxicity of the Pb and the production of free oxygen radicals. These changes lead to oxidative damage and reduced growth. The SA as a regulator of plant growth, through the enhancement of carotenoids, organic acids and the organization of antioxidant defense mechanisms, reduces the harmful effects of toxicity of Pb. In conclusion, it can be stated that among the SA treatments, the level of 100 mL L⁻¹ has the most positive effect in reducing the effects of stress of Pb on Basil plant. The application of SA increases the resistance of the plants under stress conditions. According to the results of this study, the appropriate concentration of SA for the alleviating heavy metals stress is 300 mL L⁻¹, which can increase the resistance of the plant to the stress of heavy metals.

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