Salicylic Acid Mitigates Pb Stress In Nicotiana Tabacum

Nurul Izzah Abdul Halim, Ing Chia Phang*

Department of Biotechnology, Kulliyyah of Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang *Corresponding author: ingchia@iium.edu.my

ARTICLE DETAILS

ABSTRACT

The exposure of plants to Pb stress increases the generation of reactive oxygen species (ROS) including O_2^-, H_2O_2 and ^-OH. ROS are highly reactive substances that capable of altering normal cellular metabolism via oxidative damage to membranes, proteins, and nucleic acids. Compound involves in the detoxification of Pb stress is important to reduce the toxic effect of Pb. Stress signaling compounds like salicylic acid (SA) is one of the plant defense mechanisms to mitigate Pb toxicity. This study aimed to observe the effect of SA on the morpho-physiology, accumulation of Pb content and the production of O_2^- and H_2O_2 of N. tabacum grown under Pb stress. N. tabacum was propagated and treated with SA and Pb treatments. Then, the morpho-physiology of N. tabacum was observed and measured, histochemical staining of diaminobenzidine (DAB) and nitro blue tetrazolium (NBT), and Inductively coupled plasma mass spectrometry (ICP-MS) were conducted to assesses the effect of SA on the accumulation of O_2^- and H_2O_2, and Pb content in Pb-treated N. tabacum. The finding of this study showed the application of SA improved growth parameters, diminished the accumulation of oxidative stress as well as reduced Pb content. SA might detoxify Pb, thus alleviate Pb toxicity.

1. INTRODUCTION

Lead (Pb) is one of toxic and widespread heavy metal pollutant and it becomes one of the major environmental problems. The sources of Pb include soldering, battery manufacturing, rubber and plastics industries, additive agent in fuel, as well as a pigment and drying agent in primer, paints and inks [1]. Then, Pb moves from the environment and enters the human body via food ingestion (65%), water (20%) and air (15%) [2,3]. Pb cannot be degraded, it is highly persistent and tends to enter the plants. The high accumulation of Pb in plant changes enzymatic activities, inhibits plant development and growth, and increases the production of reactive oxygen species (ROS) [3]. In order to detoxify and cope with ROS generated by Pb toxicity, plants need additional phytohormones such as salicylic acid (SA), jasmonates acid and brassinosteroids to increase the level of antioxidant activity and maintain the equilibrium between the scavenging and production of ROS. Moreover, salicylic acid was used as a signaling molecule to enhance the plant tolerance and antioxidant capacity in plants [4], thus alleviating the harmful effect of excessive ROS [5]. SA is an essential plant hormone for growth, respiration, photosynthesis, nitrate metabolism, heat production and flowering and ethylene production; it also provides protection against environmental stresses such as salinity, heavy metals and temperature [6]. The present study aimed to understand the roles of SA in protecting N. tabacum from Pb stress. The morpho-physiology of Pb-treated N. tabacum, oxidative damage, and Pb uptake were examined.

2. EXPERIMENTAL

2.1 Plant culture

Nicotiana tabacum was obtained from the Institute of Biological Science, University of Malaya (UM). The nodal part of N. tabacum was aseptically propagated in tissue culture jars containing full strength Murashige and Skoog [7; MS] medium comprising of 0.5 mg l-1 benzylaminopurine (BA; Duchefa, USA) and 0.25 mg l-1 of Indole-3-Butyric acid (IBA; Duchefa, USA). After 5 weeks, the multiplied shoots were cut into 1 cm2 segments with a sterilized blade and forceps. Roots induction was carried out in a tissue culture jar containing full strength MS medium for 3 weeks. Plant cultures were placed at 26-28 °C in a growth chamber under fluorescent light for 24 h.

2.2 SA pre-treatment and Pb treatment

After 3 weeks of root induction, the agar was aseptically removed from the N. tabacum roots and rinsed three times with sterile ultrapure H2O before transferred to sterile 10 µM SA sterile solution. After 2 days of SA pre-treatment, N. tabacum was transferred to sterile aqueous solution of 5 mM Pb for 5 days. Those without SA and Pb were served as controls (ultrapure H2O).

2.3 Morpho-physiology responses of N. tabacum

The morphology of N. tabacum was observed. Relative plant height, relative root length and relative leaf area of Pb-treated and under control treatment N. tabacum were measured by using a ruler. For the fresh weight, the samples were collected and weighed after 5 days of Pb treatment. After that, the samples were placed in a loosely closed plastic container and dried in a 60 °C laboratory dryer (FDD-720, Tech-Lab, Malaysia) for approximately 1 week to obtain the constant dry weight. All parameter were measured and calculated by subtracting final measurement (after Pb treatment) from initial measurement (before Pb treatment).

2.4 Histochemical staining of O2 and H2O2

Histochemical staining was performed according to Shi et al [8] and Xu et al [8] to observe the accumulation of superoxide (O_2^-) and (H_2O_2) in N. tabacum.

2.5 Pb content in plant material

After 1 week of SA and Pb treatments, the plant materials were collected and leaves, stems, and roots were cut using a blade and forceps. The roots were desorbed with 1 mM Na2EDTA (Duchefa, USA) for 1 min to remove surface adherent Pb and washed with ultrapure water for three times. Samples were weighed and dried until a constant dry was obtained. After that, the samples were ground and digested with 3 mL of concentrated nitric acid (65 % v/v) in 15 mL a conical tube and placed in 65°C water bath until a clear solution was obtained. The solution was diluted by adding 7 mL of ultrapure H2O. A blank (without plant material) was included to check for contamination. A standard curve was generated and Pb content was measured.

2.6 Statistical analysis

Data was analyzed using SPSS version 17.0. Three replicates from two independent experiments were used. Homogeneity of variance was tested to ensure the equality of variance between groups. All the data passed the homogeneity of variance ($P > 0.05$). Then, one-way ANOVA and post-hoc LSD test were conducted. Significant differences between untreated and treated N. tabacum were determined at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Morpho-physiology responses of SA pre-treated N. tabacum grown under Pb stress

SA was found to alleviate aluminium (Al) toxicity in rice seedling, and Sorghum bicolor varieties by reducing Al uptake, maintaining root membrane integrity, reducing ROS-induced oxidative damage and regulating the level of antioxidative enzyme activities [10]. In the present study, N. tabacum pre-treated with SA before subjecting to Pb treatment showed minimum Pb toxicity symptoms compared to N. tabacum without SA pre-treatment (Figure 1). SA pre-treated N. tabacum before subjected to Pb showed mild dehydration symptoms, compared to those without SA pre-treatment. SA might protects Pb-treated N. tabacum from dehydration by limiting the loss of water and maintains its internal water balance in the mesophyll cells (vacuoles) and spongy parenchyma cells [11]. According to Miura et al [12], soaking wheat in 100 ppm of SA for 6 h reduced the damaging effect of dehydration on cell membrane in leaves and increased the transpiration rates grown under drought stress. In the present study, SA might induce the closure of stomatal aperture in order to prevent water loss and maintain moisture content, which then reduce a harmful effect on dehydration under Pb exposure.

N. tabacum pre-treated with SA before subjecting to Pb showed the absence of yellowish symptoms on leaves compared to Pb-treated N. tabacum (Figure 1). SA might increase the chlorophyll pigments in Pb-treated N. tabacum. SA was found to increase the chlorophyll content of cucumber under drought stress [13]. SA reduces the effect of Cd and paraquat (Pq) stresses on photosynthesis in pepper (Capsicum annum L.) [14] and maize [15].

The reduction by SA on the browning of roots of Pb-treated N. tabacum was due to SA reduces the damage in the plasma membrane surface of root and stems cells by protecting N. tabacum from lipid peroxidation induced by Pb. High production of lipid peroxidation might damage the roots. SA decreased the membrane lipid peroxidation in N. tabacum root which increases by Pb stress. For example, SA was found to protect barley roots from lipid peroxidation induced by Cd [16].

After 5 days of Pb treatment, N. tabacum treated with Pb showed maximal growth inhibition in plant height, root length, and fresh weight, in comparison to the controls and SA pre-treated N. tabacum before subjected to Pb treatment (P <0.05; Table 1). SA pre-treated N. tabacum before subjected to Pb had similar growth pattern with control and SA treatments (P >0.05; Table 1). The dry weight and leaf area of N. tabacum did not differ significantly in all treatments (P >0.05). SA plays an important role in plant growth and development. It acts as a plant growth regulator to increase the crop yields and protect plants against heavy metal toxicity. N. tabacum that had been pre-treated with SA before being exposed to Pb were taller, had longer roots, and greater fresh weight in comparison to Pb-treated N. tabacum (P <0.05; Table 2). No significant difference was detected between SA+Pb with control and SA pre-treatment (P >0.05). This clearly indicates that, SA involved in the cell wall expansion and relaxation mechanisms in maintaining the osmotic transport of the cell in order to maintain cell wall synthesis, form and thickness [10]. For examples, SA increased growth of maize, wheat (Triticum aestivum L.) and barley grown under salinity stress [17]. In this study, the accumulation of SA in the aerial part of Pb-treated N. tabacum might reduce the accumulation of Pb in the aerial part of N. tabacum, thus Pb did not interfere with the growth of N. tabacum.

Figure 1 Morphological observation on the effect of SA on N. tabacum grown in Pb solution for 5 days: (a) control, (b) SA, (c) Pb and (d) SA+Pb. Scale bar: 1 cm
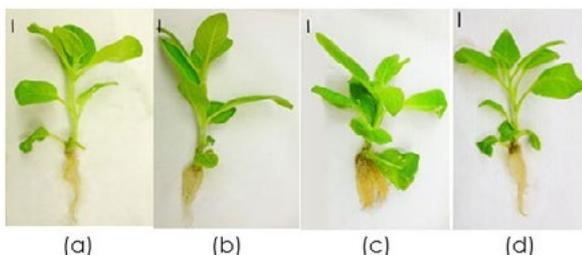
Similarly, free SA and glucosyl SA accumulate in Arabidopsis shoots as well as in wheat and grape berry under chilling stress [12]. Therefore, the result in this present study signify the role of SA in alleviating the Pb stress and suggest that SA could be used as a potential growth regulator to improve plant growth, under Pb stress. SA pre-treatment before subjected to Pb treatment was found to increase the root length in comparison to Pb-treated N. tabacum (P <0.05; Table 2). According to a previous study, the large supply of carbohydrate must flow via the phloem to the apical root zones in order to maintain and support the root cell elongation and division [17]. SA

treatment was found to increase the accumulation of soluble carbohydrates in roots and shoots of wheat grown in response to copper (Cu) stress. Similarly, the treatment of wheat plants and tomato with SA increased the level of cell division within the apical meristem of seedling roots against salinity stress. Fresh weight of SA pre- treatment N. tabacum before being treated with Pb was significantly higher compared to Pb-treated N. tabacum (P <0.05; Table 2). The protective action of SA during Pb stress in many plants was demonstrated by the accumulation of different osmolytes such as ABA, alcohol and proline. It is also responsible for osmotic adjustment to reduce cell water deficit to prevent dehydration. Osmotic adjustment causes reduction of adverse effect of plant water deficit in response to abiotic stress. It is likely, SA reduces the damaging effects caused by water deficit and maintains normal metabolism for growth in Pb-treated N. tabacum. Dry weight and leaf area did not differ significantly for all the treatments (Table 2; P >0.05). This indicates that the body mass for all the N. tabacum treated with different treatments had similar body mass after all the water has been removed. SA pre-treated N. tabacum before subjected to Pb appeared to be fresh and healthy, showing that SA has the ability to alleviate the negative effect of Pb in plants. The application of SA also reduced the translocation of Pb to the leaves and stems of Pb-treated N. tabacum. Pb was retained mostly in roots of N. tabacum.

Table 1 Effect of SA on the toxicity symptoms of Pb-treated N. tabacum

	Leaves		Roots		Plants conditions	
	Yellowing	Thickening	Browning	Thickening	Wilting	Dehydration
Control	-	-	-	-	-	-
SA	-	-	-	-	-	-
Pb	+	++	+++	++	++	++
SA + Pb	-	-	++	+	+	+

Table 2 Effect of SA treatment on relative plant height (RPH), (b) relative root length (RRL), relative leaf area (RLA), relative fresh weight (RFW), and (e) dry weight (RDW) in Pb-treated N. tabacum. Differents letters indicate significant difference between treatments

	RPH	RRL	RLA	RFW	RDW	TI, %
Control	0.80 ± 0.1^a	0.30 ± 0.09^a	0.05 ± 0.02^a	0.18 ± 0.02^a	0.06 ± 0.007^a	100
SA	0.60 ± 0.04^a	0.30 ± 0.04^a	0.03 ± 0.01^a	0.23 ± 0.013^a	0.04 ± 0.003^a	72
Pb	0.10 ± 0.02^b	0.10 ± 0.02^b	0.03 ± 0.01^a	-0.36 ± 0.02^b	0.04 ± 0.005^a	68
SA+Pb	0.60 ± 0.08^a	0.30 ± 0.04^a	0.05 ± 0.01^a	0.03 ± 0.01^a	0.06 ± 0.008^a	98

3.2 The Pb content in SA pre-treated N. tabacum grown under Pb stress

The Pb content of SA pre-treated N. tabacum exposed to Pb stress was significantly lower in stems and leaves, compared to N. tabacum treated with Pb alone (Table 3). The present study found that SA pre-treatment limited the transport and mobility of Pb from roots to shoots. It has been reported that SA tends to increase the biosynthesis of SA in order to prevent the plant from recognizing heavy metals stress by limiting the uptake and accumulation of heavy metals in roots [18]. The increase biosynthesis of SA increases the production of proline, which acts as a metal chelator [19, 9]. Proline appears to chelate heavy metals ion in cytoplasmic region, which is essential for detoxification of heavy metals [19]. SA reduces the concentration of heavy metals ions in the cytoplasm by preventing the metal from being transported across the plasma membrane [19]. There is no difference between the accumulation of Pb in the roots of Pb and SA+Pb treated N. tabacum. Similarly, sodium nitroprusside (SNP) pre-treatment did not reduce the accumulation of Pb in Arabidopsis thaliana [20]. This might be due to the Pb reduce the total content of SA in roots and this was accompanied by the elongation of root length. Similarly, seed pre-sowing treatment reduced the SA content and SA biosynthesis in root and shoot of Triticum aestivum L [21].

In addition, SA pre-treatment might increase SA biosynthesis and enhanced the detoxification of Pb in Pb-treated N. tabacum. SA increased the phytochelatin (PCs) activities in plants in response to heavy metal stress [22]. PCs are cysteine-rich peptides, which are enzymatically synthesized [23, 24, 25]. PCs blocks the entry of heavy metals into cytoplasm via chelation process, either by increasing binding of metal ions to the cell wall or by reducing the uptake of heavy metal through modified ion channels, or by pumping the metal out of the cell. PCs helps Salvinia minima to cope with Pb stress by the binding of PCs to Pb ions leading to sequestration of Pb ions in plants and thus served as an important component of the detoxification mechanism in plants. It is likely that SA could increase the activities of PCs to reduce the transport of Pb to the aerial part of N. tabacum thus Pb did not interfere with the growth of N. tabacum, Pb content in the roots of Pb-treated N. tabacum did not differ significantly in comparison to SA pre-

treated N. tabacum before being subjected to Pb (Table 3; P >0.05). Pb was predominantly accumulated in the roots of N. tabacum and Pb concentration was higher in the roots than the aerial parts. Similarly, the uptake and accumulation of Pb in Allium sativum was measured by inductively coupled plasma atomic emission spectrometry (ICP-AES), indicating Pb accumulated mainly in roots and Pb contents in bulbs and shoots were much lower than root [26]. According to [27], approximately 90% of Pb is localized and accumulated in the insoluble fraction of cell walls and nuclei in roots of plants. For examples, Pb accumulated in the roots of a number of species of the Brassicaceae family and several crops species includes Z. mays, Pistia stratiotes, Brassica rapa, Arabidopsis thaliana and water hyacinth [23, 20]. This was because the roots were the first organs to encounter with toxic Pb ions [27]. The retention of Pb in roots is due to the binding of Pb ion to ion-exchange sites located on the cell wall and extracellular precipitation. The accumulation of Pb in root takes place by binding with polysaccharides, with organic acids and cell walls in the roots and xylem vessels and thus Pb might become immobile in roots [20].

Table 3 Effect of SA on Pb content in leaves, stems and roots of Pb-treated N. tabacum. Differents letters indicate significant difference between treatments.

Treatment	Leaves	Stems	Roots
Control	0 ± 0.00^a	0 ± 0.00^a	0 ± 0.00^a
SA	0 ± 0.00^a	0 ± 0.00^a	0 ± 0.00^a
Pb	33 ± 11^b	1700 ± 190^b	8240 ± 964^b
SA+Pb	4 ± 2.60^{ab}	703 ± 174^c	9496 ± 1490^b

3.3 Accumulation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in SA pre-treated N. tabacum

The dark blue spot of O_2- and the dark brown of H_2O_2 were observed and compared between N. tabacum subjected to different treatments (Figure 2). Higher amounts of O_2- and H_2O_2 were seen in Pb-treated N. tabacum in comparison to other treatments. NBT reacts with O_2- to produce a dark blue insoluble formazon compound. In the present study, the higher accumulation of Pb in leaves of Pb-treated N. tabacum induced the high accumulation of O_2- and H_2O_2. O_2- and H_2O_2 may inactivate enzyme by oxidizing their thiol groups and travel across the membrane, and they may also act as messenger in the stress signaling molecules [28]. The high accumulation of O_2- and H_2O_2 leads to cell membrane lipid peroxidation, causing membrane damage and leakage of electrolytes. For examples, cold stress enhanced the production of malonic dialehyde (MDA) and relative electrolyte leakage in jasmine leaves, confirming the presence of oxidative stress [2]. In addition, H_2O_2 is subsequently converted into hydroxyl radical (HO·), which is a highly reactive oxidant in the family of ROS [29]. O_2- and H_2O_2 can interfere and alter the functions of proteins, lipids and DNA [30] by reacting with them, causing oxidative damage and impairing the normal cellular functions [31]. SA pre-treatment partially abolished O_2- and H_2O_2 formation in Pb-treated N. tabacum (Figure 2). The exposure of plant to cadmium increases the biosynthesis of SA in wheat varieties [32]. In N. tabacum, SA is synthesized via the formation of benzoic acid from cinnamic acid pathways [18]. SA is converted to methylsalicate (MeSA) in the leaves cuticle, which is an inactive form of SA. Then, MeSA will be converted to SA upon the exposure to Pb. This allows SA to maintain the trans-membrane redox balance, by increasing the activity of antioxidant enzymes and proline. SA biosynthesis increases the accumulation of proline in response to heavy metals. Proline appears to be involved in the chelation of excess cytoplasmic metal ions, which shows a preference for nitrogen or oxygen coordination. Due to its zwitterionic and high hydrophilic character, proline may act as protein stabilizer, metal chelator, free radical scavenger and provide protection to the enzyme and biomolecules [29]. SA pre-treatment decrease the oxidative stress accumulation in rice roots and seedlings grown under Cd, Mn and Pb stress [33, 34], Triticum polonicum L. grown under manganese (Mn) toxicity [35], and jasmine leaves in cold stress [2]. In this study, SA treatment managed to mitigate the oxidative stress in Pb-treated N. tabacum.

4. CONCLUSION
In conclusion, Pb adversely affects the morphology and growth of N. tabacum, increased Pb accumulation in N. tabacum, enhanced the production of O_2- and H_2O_2. However SA pre-treatment was found to alleviate the Pb toxicity in N. tabacum. SA pre-treated N. tabacum before subjected to Pb seems to have a better growth with minimum Pb toxicity symptoms. SA decreased Pb accumulation in the aerial part of Pb-treated N. tabacum and reduced the accumulation of H_2O_2 and O_2-, compared to those without SA pre-treatment. The present study demonstrated that SA appeared to be a beneficial phytohormone to enhance N. tabacum tolerance to Pb and protects N. tabacum from Pb-induced oxidative damage.

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