

Role of Salicylic Acid in Alleviating Cobalt Toxicity in Wheat (*Triticum aestivum* L.) Seedlings

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Received: February 2, 2019

Accepted: May 7, 2019

Online Published: July 15, 2019

doi:10.5539/jas.v11n10p112

URL: <https://doi.org/10.5539/jas.v11n10p112>

Abstract

Heavy metals pollution of soils become the most serious environmental problem resulted in decreased soil fertility and crop yield losses. Cobalt (Co), as a beneficial element, can be a contaminant in soils due to agricultural additives or metal refineries, it causes irreversible damage to several physiological and metabolic constituents at higher concentrations. In this investigation we study the effects of different Co concentrations (0, 50, 150, 250, 350 μM as CoCl_2) on growth and physiological processes in wheat (*Triticum aestivum*) plants and discuss the role of spraying with salicylic acid (0.5 mM) for ameliorating effect of cobalt toxicity in wheat leaves. Cobalt causing a significant reduction in fresh and dry biomass, shoot height, water content and total photosynthetic pigments. This was accompanied with accumulation of H_2O_2 and MDA contents and induction of some antioxidant enzymes activities. There was an increase in SOD activity with increasing Co concentration then decline, a significant increase in APX and PPO activities while CAT was significantly declined. Spraying wheat seedlings with salicylic acid can be recognized to significantly increase in all tested growth parameters and that was attributed to decrease, to some extent, the accumulation of H_2O_2 and MDA and improve the activity of antioxidant enzymes.

Keywords: antioxidant enzymes, cobalt, heavy metals pollution, salicylic acid, *Triticum aestivum*

1. Introduction

Abiotic stresses, such as heavy metals, salinity, ozone, UV-B radiation, high or low temperatures and drought, are among the most challenging threats to agricultural system and economic yield of crop plants. Heavy metals pollution is one of the most serious environmental problems which are dominantly found in industrial and sewage wastes (Jayakumar et al., 2007; Abdul Jaleel et al., 2009; Liu et al., 2014). Beneficial elements are necessary for the normal metabolic functions of particular plants, but at higher concentrations, these metals are toxic and may severely interfere with physiological and biochemical functions (Jayakumar and Vijayarengan, 2006; Imtiyaz et al., 2014). Cobalt (Co), as a beneficial element, can be a contaminant in soils due to agricultural additives or metal refineries (Bakkaus et al., 2005; Zaborowska et al., 2016). It causes irreversible damage to several physiological and metabolic constituents at higher concentrations (Abdul Jaleel et al., 2009; Singh et al., 2010). Karuppanapandian and Kim (2013) and Abdal Dayem et al. (2017) reported that excess Co induced ROS that caused growth inhibition and enhanced lipid peroxidation, in addition to DNA degradation.

It is known that Plants under stress activate various powerful defense mechanisms to protect themselves from the harmful effect of oxidative stress. ROS scavenging is one among the common defense response against abiotic stresses including enzymatic and non-enzymatic antioxidants (Vranova et al., 2002; Singh et al., 2010; Karuppanapandian & Kim, 2013).

Salicylic acid (SA), a hormone-like substance, acts as a signaling molecule and plays a vital role in the regulation of plant growth and development, such as seed germination, flowering, and fruit ripening and acclimation responses to abiotic stressors (Alam et al., 2013; Alavi et al., 2014; Alamri et al., 2018). Salicylic acid application caused a partial protection against cadmium toxicity in barley seedlings (Metwally et al., 2003) and alleviated the heavy metal-induced membrane degradation in rice seedlings (Mishra & Choudhuri, 1999). Wang et al. (2004) provided evidence that treatment with SA could modulates the Al-induced oxidative damage in root tips.

Our research aimed to show the effects of Co stress on growth and physiological processes in wheat (*Triticum aestivum*) seedlings with specific emphasis on the activity of antioxidant enzymes, and the role of salicylic acid in ameliorating the effect of cobalt toxicity in wheat leaves.

2. Materials and Methods

2.1 Plant Material, Growth Conditions and Treatments

Wheat grains (*Triticum aestivum* L) Sakha 93 were obtained from the Agricultural Research Center, Giza, Egypt. They were surface sterilized with 2% sodium hypochlorite for 10 min, washed with distilled water, then soaked for 24 h at 25 °C in aerated distilled water. Twenty soaked grains were transferred to pots (15 cm diameter) filled with previously acid-washed quartz sand and placed in a completely randomized design in an environmentally controlled growth chamber under a 16/8 h photoperiod at an irradiance of about 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (cool white fluorescent tubes) and 28/21 \pm 2 °C light/dark temperature for 15 days. The pots were irrigated with 0, 50, 150, 250 and 350 $\mu\text{mol CoCl}_2$ every two-day interval. To prevent Co accumulation, irrigation with distilled water was done between intervals. After 8 days from starting experiment, the seedlings were sprayed with 0.5 mM salicylic acid (SA) every two day. Homologous seedlings were collected at the end of experimental period (15 days), washed, blotted gently, dissected to root and shoots and saved for determination of fresh, dry biomass and chemical analyses. Other fresh samples were saved in liquid N₂ for enzymes assay.

2.2 Determination of Fresh, Dry Biomass and Shoot Height

The root and shoot of homologous seedlings (three replicates) were taken and weighed as fresh biomass. The dry biomass was determined after drying the samples in an oven at 60 °C till constant weight. Shoot height were estimated to nearest centimeters.

2.3 Determination of Photosynthetic Pigments

The photosynthetic pigments chlorophyll a, b (Chl. a, Chl. b) and carotenoids (carot.) were determined following N, N-dimethyl formamide (DMF) method described by Inskeep and Bloom (1985).

2.4 Determination of H₂O₂ and Malondialdehyde (MDA) Contents

Hydrogen peroxide content was determined according to Velikova et al. (2000). The content of H₂O₂ was calculated by comparison with a standard calibration curve using different concentrations of H₂O₂. The level of lipid peroxidation was measured according to the thio-barbituric acid (TBA) test, which determines the malondialdehyde (MDA) as the end product of the lipid peroxidation reaction (Heath & Packer, 1968).

2.5 Assays of Antioxidant Enzymes

2.5.1 Enzymes Extraction

These were carried out according to Azevedo Neto et al. (2006).

2.5.2 Activity Measurement

Superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium chloride (NBT) as described by Giannopolitis and Ries (1977). One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photo-reduction rate.

Catalase (CAT, EC 1.11.1.6)

Catalase activity was measured according to the method of Azevedo Neto et al. (2006). The decrease of H₂O₂ was monitored at 240 nm and quantified by its molar extinction coefficient (36 M⁻¹ cm⁻¹).

Ascorbate peroxidase (APX, EC 1.11.1.11)

Ascorbate peroxidase activity was assayed according to Nakano and Asada (1981). Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mM⁻¹ cm⁻¹). Taking into consideration that 2 mol ascorbate are required for reduction of 1 mol H₂O₂.

Polyphenol oxidase (PPO, EC 1.10.3.1)

The polyphenol oxidase activity (PPO) was assayed as the method described by Kumar and Khan (1982). One unit (U) is defined as the amount of purpurogallin formed, which raised the absorbance by 0.1 per minute under the assay condition.

2.6 Determination of Salicylic (SA) Acid Content

High performance liquid chromatography (HPLC) method was validated for simultaneous determination of salicylic acid in a plant tissues according to Sawyer and Kumur (2003).

2.7 Statistical Analysis

All data were expressed as means of triplicate samples. Comparisons of means were performed using SPSS 20.0 software. Data were subjected to a one-way analysis of variance (ANOVA), and the mean differences were compared by lowest standard deviations (LSD). Comparisons with $P \leq 0.05$ were considered significantly different.

3. Results

Various cobalt levels significantly decreased fresh and dry biomass in shoot and roots of wheat plants. The decrease of fresh and dry biomass in 350 μM Co-treated shoots were 77% and 44% respectively compared to untreated control. For roots, this decline was 80% and 63% respectively. Water content in shoots and roots as well as shoot height were also reduced in response to Co treatment (Table 1). The severe reduction of growth parameters of Co-treated plants was markedly less pronounced in SA-sprayed Co-stressed ones. The increase of fresh matter in shoots and roots of 250 μM Co-treated SA-sprayed plants was 36% and 19% respectively compared to non-SA sprayed plants. These values in dry matter were 28% and 16% respectively.

Table 1. Effects of spraying with salicylic acid (0.5 mM) on the fresh, dry biomass, water content and shoot height of 15 day-old wheat seedlings growing under different CoCl_2 concentrations

Treatments		Shoots				Roots		
CoCl_2	SA	F.M.	D.M.	$\text{H}_2\text{O}\%$	S.H.	F.M.	D.M.	$\text{H}_2\text{O}\%$
$\mu\text{mol/L}$	mM	----- g^{-1} Plant -----						
Control	0.0	2.93±0.21	0.52±0.04	82±5.47	18.5±1.54	1.37±0.10	0.3±0.03	83±6.38
50	0.0	2.62±0.22	0.5±0.04	81±5.06	19.2±1.28	1.45±0.12	0.31±0.02	79±5.64
150	0.0	1.85±0.12	0.43±0.03	77±5.50	14.4±1.11	0.85±0.06	0.22±0.02	74±5.69
250	0.0	1.17±0.08	0.32±0.03	68±4.25	9.7±0.65	0.52±0.03	0.19±0.02	63±4.85
350	0.0	0.68±0.04	0.29±0.02	57±4.07	6.1±0.38	0.28±0.02	0.11±0.01	50±3.33
Control	0.5	3.03±0.20	0.53±0.03	83±6.38	20.5±1.58	1.89±0.12	0.33±0.03	83±5.93
50	0.5	2.89±0.21	0.54±0.05	81±5.06	21±1.31	1.78±0.15	0.32±0.02	82±5.13
150	0.5	2.42±0.20	0.48±0.04	83±6.38	16.5±1.10	1.34±0.12	0.26±0.02	80±5.33
250	0.5	1.59±0.14	0.41±0.03	74±6.17	14.3±1.30	0.62±0.05	0.22±0.02	76±6.33
350	0.5	0.96±0.09	0.37±0.02	61±3.81	10.8±0.72	0.51±0.03	0.21±0.02	66±4.71
P		0.0035*	0.037*	0.0041*	0.0013*	0.011*	0.036*	0.006*
LSD		0.62	0.14	10.00	3.55	0.42	0.15	15.00

Note. SA = salicylic acid; F.M. = fresh matter; D.M. = dry matter; S.H. = shoot height; $\text{H}_2\text{O}\%$ = percent of water content. Values are mean±SD (n = 3); * Difference is significant at $p \leq 0.05$.

The decline of photosynthetic pigment contents also indicated the inhibitory effect of Co on photosynthetic machinery in wheat leaves. The suppression in Chl. a and Chl. b contents was 81% and 71% respectively, in 350 μM Co-treated plants in comparison to control (Table 2). Spraying Co-treated plants with SA resulted in an increase or improve the photosynthetic pigments content compared to SA-untreated plants. The increase of Chl. a and b levels in 350 μM Co-stressed plants and sprayed with SA was 47% and 54% respectively versus SA-untreated ones. Although, carotenoids content in Co-stressed leaves was significantly decreased compared to those of untreated plants, spraying Co-stressed plants with SA insignificantly changed the carotenoid contents compared to the SA non-sprayed ones (Table 2).

It was seen that SA content in Co-stressed leaves of SA non-sprayed or sprayed wheat plants were significantly increased as concentration gradient, but the attained values of later were markedly higher than those of the former (Table 3). At 50 and 250 μM Co, SA content in the SA non-sprayed leaves was 1.9- and 2.8-fold respectively of control, whereas in those SA-sprayed was 2.2- and 3.5-fold respectively.

Table 2. Effects of salicylic acid (0.5 mM) on the pigments content of leaves of 15 day-old wheat seedlings growing under different CoCl₂ concentrations

Treatments		Chl. a	Chl. b	Carot.	Total
CoCl ₂	SA				
μmol/L	mM	----- mg g ⁻¹ F.M. -----			
Control	0.0	3.11±0.28	1.40±0.13	0.65±0.05	5.16±0.32
50	0.0	3.03±0.25	1.45±0.10	0.85±0.07	5.33±0.46
150	0.0	2.47±0.15	0.84±0.08	0.48±0.04	3.79±0.24
250	0.0	0.87±0.07	0.74±0.06	0.31±0.03	1.92±0.12
350	0.0	0.6±0.05	0.41±0.03	0.26±0.02	1.27±0.11
Control	0.5	3.15±0.26	1.58±0.11	0.61±0.05	5.34±0.36
50	0.5	3.19±0.20	1.56±0.11	0.92±0.05	5.67±0.38
150	0.5	2.79±0.20	1.03±0.06	0.54±0.04	4.36±0.27
250	0.5	1.76±0.12	0.95±0.06	0.43±0.04	3.14±0.21
350	0.5	0.88±0.06	0.63±0.05	0.32±0.03	1.83±0.11
P		0.0021*	0.032*	0.028*	0.0035*
LSD		1.00	0.50	0.22	1.10

Note. Chl. a = chlorophyll a; Chl. b = chlorophyll b; Carot. = carotenoids; F.M. = fresh matter; SA = salicylic acid. Values are mean±SD (n = 3); * Difference is significant at $p \leq 0.05$.

Table 3. Effect of spraying with salicylic acid (0.5 mM) on salicylic acid contents of leaves of 15 day-old wheat seedlings growing under different CoCl₂ concentrations

Treatments		SA contents
CoCl ₂	SA	
μmol/L	mM	μg g ⁻¹ F.M.
Control	0.0	128±9.85
50	0.0	245±15.31
150	0.0	354±22.13
250	0.0	354±22.13
350	0.0	470±29.38
Control	0.5	146±10.43
50	0.5	326±27.17
150	0.5	420±28.00
250	0.5	514±46.73
350	0.5	594±54.00
P		0.001*
LSD		75.00

Note. SA = salicylic acid. Values are mean±SD (n = 3); * Difference is significant at $p \leq 0.05$

There was a significant increase of H₂O₂ and MDA contents in wheat leaves according to the increase of Co concentrations (Figure 1). At 250 μM Co the H₂O₂ and MDA content in leaves was 3.8- and 4.0-fold respectively compared to Co-untreated control (Figure 1). Spraying control wheat leaves with SA significantly decreased H₂O₂ and MDA contents compared to untreated plants. In addition, spraying with SA exhibited a significant decline of H₂O₂ and lipid peroxidation in Co-treated leaves. The reduction of H₂O₂ and MDA contents in 250 μM Co-stressed leaves SA-treated was 23% and 30 % compared to those SA-untreated plants, respectively.

Cobalt resulted in an induction of the antioxidant enzyme activities (Figure 2). The SOD activity increased up to 150 μM Co then steady decline. APX and PPO activities were markedly increased with increasing Co levels. On the other hand, CAT activity was continuous decrease up on all Co concentrations. Spraying Co-treated plants with SA mainly alleviated the inhibitory effect of Co *via* enhancement antioxidant activities. At 350 μM Co the SOD, CAT, APX and PPO activities in SA-sprayed leaves was 1.4-, 8.3-, 2.2- and 2.1-fold respectively compared to SA unsprayed plants.

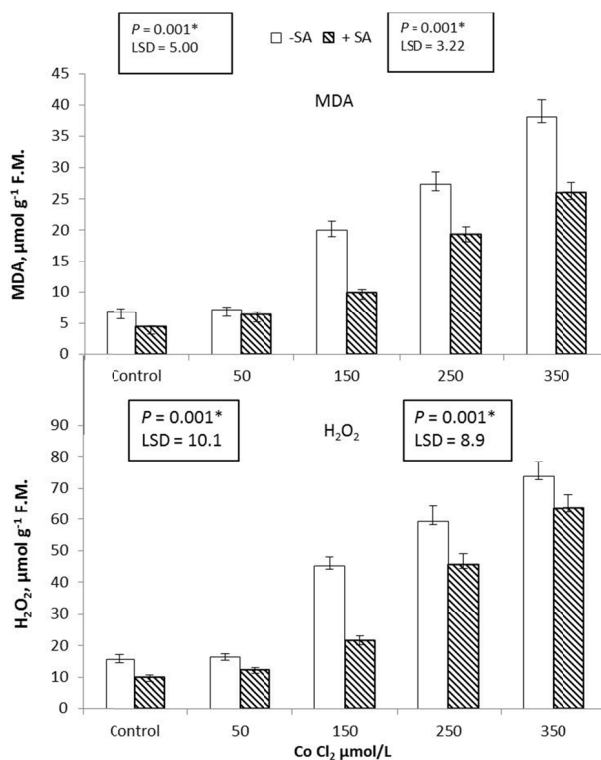


Figure 1. Effects of spraying with salicylic acid (0.5 mM) on malondialdehydes (MDA) and hydrogen peroxide (H_2O_2) contents of leaves of 15 day-old wheat seedlings growing under different CoCl_2 concentrations

Note. MDA= malondialdehydes; H_2O_2 = hydrogen peroxide. Values are mean±SD (n = 3); * Difference is significant at $p \leq 0.05$.

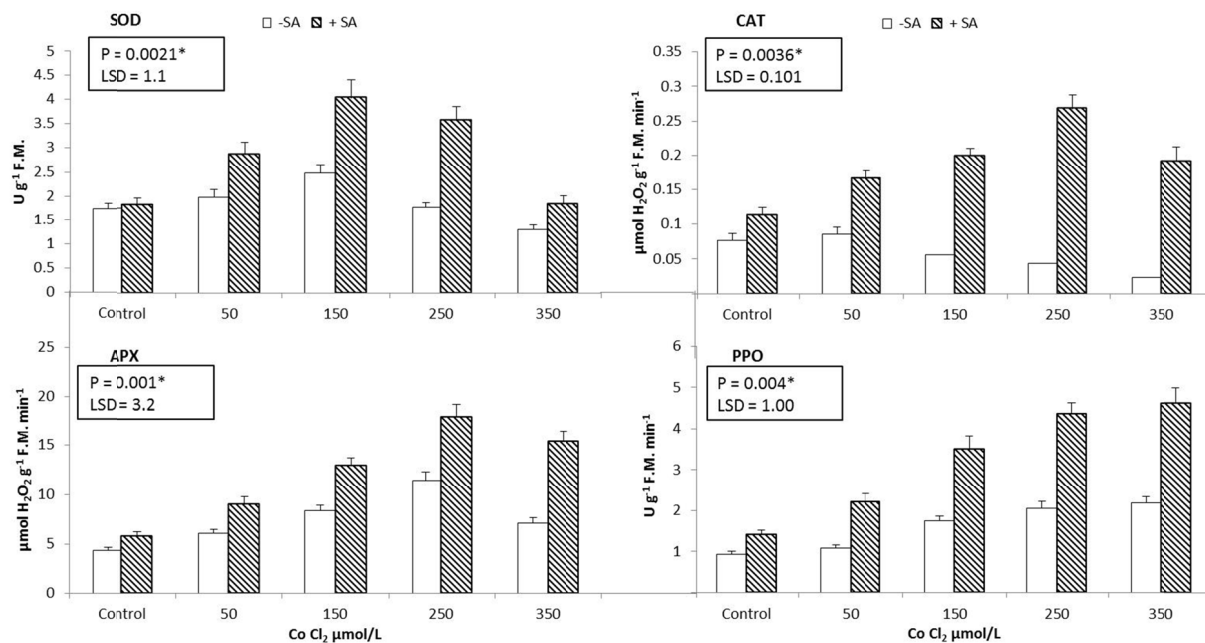


Figure 2. Effects of spraying with salicylic acid (0.5 mM) on antioxidant enzyme activities of leaves of 15 day-old wheat seedlings grown under different CoCl_2 concentrations

Note. SOD = superoxide dismutase; CAT = catalase; APX = ascorbate peroxidase; PPO = polyphenol oxidase. Values are mean±SD (n = 3); *Difference is significant at $p \leq 0.05$.

4. Discussion

Wheat plants imposed to various Co concentrations exhibited a significant reduction of growth, measuring by fresh and dry biomass and shoot height (Table 1). Similar suppression in plant growth was shown previously in several plants including *Vigna mngo* (Jayakumar & Vijayarangan, 2006), *Raphanus sativus* (Jayakumar et al., 2007) and cucumber plants (Gad et al., 2008). Excessive Co treatment could suppress the root growth by the decrease of cell division or elongation resulting in limitation of water and nutrients absorption (Gad et al., 2008; Singh et al., 2010). Thus, the increase of H₂O₂ and MDA contents accompanied with increase of Co accumulation-in this study-might reflect the disturbance of plasma membranes integrity and degradation of macromolecules such as proteins, RNA and DNA (Shao et al., 2008; Gill & Tuteja, 2010) leading to the decrease of water and nutrients uptake; hence resulting in the decline of wheat growth.

Salicylic acid spraying significantly increased growth parameters of wheat plants. Similarly, the growth promoting effect of SA against various heavy metals stress has been demonstrated in several plant species (Metwally et al., 2003; Wang et al., 2004; Belkadhi et al., 2014). Thus, the increase of biomass of Co-stressed wheat plants sprayed with SA might be explained by enhancement of cell divisions and enlargement (Klessig & Malamy, 1994) and suppression of H₂O₂ and MDA contents which reflect the improvement of plasma membranes integrity and photosynthetic apparatus. Chen et al. (2007) and Zengin (2014) reported that SA pretreatment decreases membrane lipid peroxidation in Pb-exposed rice and Cu-exposed bean seedlings, respectively.

Krantev et al. (2008) have stated that heavy metals pollution resulted in a marked decrease of photosynthetic pigments content and photosynthetic rate. In accordance with these views, Chl. a, b and total photosynthetic pigment contents in wheat leaves significantly decreased in a concentration dependent manner of Co. El-Sheekh et al. (2003) suggested that Co might increase chlorosis in plants *via* preventing the incorporation of Fe in protoporphyrin molecules and interference with enzyme proteins responsible for chlorophyll biosynthesis, resulting in impairment of chlorophyll synthesis. In addition, Singh et al. (2010) reported that heavy metals toxicity might result in nutrient deficiency. Therefore, depression of chlorophyll pigments synthesis in wheat plants could be attributed to Co causing a decline of essential nutrient absorption such as nitrogen, phosphorus, potassium and iron, beside the prevention of proporphyrin molecules induction. Moreover, degradation of thylakoid and chloroplast membranes by generated H₂O₂ might be considered as one of the factors for suppression of chlorophylls content in SA sprayed and non-sprayed wheat leaves. On the other hand, carotenoids were less affected in Co-treated wheat leaves. Similar results were obtained by El-Sheekh et al. (2003) and Imtiyaz et al. (2014).

Salicylic acid treatment has a beneficial effect on photosynthetic apparatus in wheat plants under Co stress. The results in this article pointed to photosynthetic pigments content was significantly higher in Co-treated wheat plants sprayed with SA compared to those non-sprayed ones. These results suggested that SA might played different roles based on the protection of the photosynthetic machinery from oxidative damage as shown by decrease of the H₂O₂ and MDA (Figure 1), as well as the induction of photosynthetic pigments *via* the increase of the uptake of essential nutrients. Zengin (2014) reported that SA treatment protects the photosynthetic pigments and reduces plasma membrane destruction in Cu-exposed bean seedlings. Similarly, the protective role of SA in shifting off the inhibitory effect of heavy metals has been shown by several authors (Liu et al., 2011; Belkadhi et al., 2014; Alamri et al., 2018).

Heavy metal stress has been reported to induce oxidative damage of various plant cell components due to generation of ROS (Jayakumar et al., 2007; Karuppanapandian & Kim, 2013; Abdal Dayem et al., 2017). A certain H₂O₂ concentration can play a role as a secondary messenger for activating the specific tolerance related genes in the stressed organisms, while the increase of H₂O₂ accumulation may be considered as a toxic agent (Shao et al., 2008; Herrera-Vásquez et al., 2015). In this study, there was a significant increase of H₂O₂ and MDA contents in Co-stressed wheat leaves, and that was accompanied with a marked decrease of growth. Similar results have been shown earlier in several plant species imposed to heavy metals stresses (Karuppanapandian & Kim, 2013; Abdal Dayem et al., 2017; Alamri et al., 2018). Foliar spraying with SA resulted in a significant decrease of H₂O₂ and MDA contents in leaves of Co-stressed wheat plants, compared to non-sprayed ones. These observations in agreement with previous reports in rice (Jing et al., 2007), maize (Krantev et al., 2008). In order to shift off the oxidative damage by generated ROS, plants evolve complex enzymatic and non-enzymatic defense mechanism. The results-in this study-showed that Co treatment increased the activity of SOD, APX and PPO in wheat leaves revealing the activation of enzymatic antioxidant system for detoxification of generated superoxide (O^{•-2}), ionic oxygen (O[•]) and H₂O₂. In addition, foliar application of SA markedly up-regulated the activity of SOD, PPO (O^{•-} and O[•] eliminating enzymes) and APX (H₂O₂-scavenging

enzyme) activities in wheat leaves. These observations are in a good agreement with those shown by several authors (Karuppanapandian and Kim, 2013; Luo et al., 2014; Alamri et al., 2018) under heavy metal stress.

In the present study, foliar SA spraying significantly activated CAT activity in Co-treated wheat leaves. These results might be related to the binding of SA with enzyme protein (Chen et al., 1993). Jay et al. (1999) reported that salicylate-iron complex with SOD enzyme may enhance the dismutation of generated superoxide. While, Alamri et al. (2018) reported that SA can directly scavenge generated ROS and/or indirectly induce the redox potential of antioxidant enzymes.

5. Conclusion

The results of this study indicate that foliar SA spraying of Co-treated wheat leaves significantly increase all tested growth parameters and that was attributed to decrease, to some extent, the accumulation of H₂O₂ and MDA and might reflect its role for enhancement the activity of antioxidant enzymes (*via* activation of SOD and PPO) to minimize the oxidative damage induced by Co.

Acknowledgements

We would like to express our profound gratefulness to Prof. Dr. Nabil El-Sayed Saber, for his inspiring guidance and his kind constructive advice.

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