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Treatment with polyamines alleviates the effects of concomitantly applied aluminum in sunflower (*Helianthus annuus* L.) leaves

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Abstract

Contamination of agricultural soils with heavy metal is a significant risk for the environment. Many substances are reported to alleviate the toxic effects of heavy metals such as polyamines. The aim of this study is to examine whether the toxic effects of 0.1 mM aluminum, which is previously detected in sunflower leaves, might be alleviated with 0.1 mM putrescine, spermine or spermidine and to compare the effects of putrescine, spermine and spermidine in the ameliorating process. Chlorophyll *a*, carotenoid and anthocyanin content increased after putrescine, spermine and spermidine treatment under aluminum toxicity. However, chlorophyll *b* and total chlorophyll content only increased after spermine treatment. Intense accumulation of reactive oxygen species under aluminum toxicity decreased after putrescine, spermine and spermidine treatment while the spermine showed the maximum decrease. Superoxide dismutase enzyme activity and hydrogen peroxide content increased after putrescine, spermine and spermidine treatment while the spermine showed the maximum increase. Besides, catalase enzyme activity increased only after spermine treatment. Results showed that 0.1 mM putrescine, spermine and spermidine increased the 0.1 mM aluminum toxicity tolerance of sunflower leaves by modulating the reactive oxygen species detoxification metabolism. Spermine was the most effective polyamine in improving the aluminum tolerance.

Keywords: Aluminum toxicity, leaves, polyamines, reactive oxygen species**,** sunflower

1. Introduction

Sunflower (*Helianthus annuus* L.) is a unique member belongs to Asteraceae family. It is one of the most valuable oilseed crops and, due to its high economic value it is widely cultivated all over the world including contaminated agricultural soils by heavy metals (HM). Contamination of agricultural soils with HMs is a significant risk for the environment. Particularly, sunflower has the ability to amass HMs in its harvesting part. Therefore, it is one of the plants which are under the risk of HM toxicity [1].

Aluminum (Al) is the third most copious element in the earth's crust. Although it is the amplest metallic element in soil, it is not toxicant for the plants in near-neutral or alkalescent soil. However, it is toxic for plants in acidic soils. Considering the almost 70% of the world's agricultural areas have acidic soil, Al is considered one of the significant factors which restrict plant development [2]. Due to root is the initial target of Al toxicity, the most of the studies have been carried out in the root. However, Al is transported to different plant tissues such as stems or leaves after absorption by the roots. So, the toxicity of Al affects not only the roots of plants, but also the aerial part such as leaves [3]. Nevertheless, knowledge concerning the responses to Al toxicity of leaves is still open to improvement.

Researchers have been reported that determination of HM toxicity levels can be monitored by alterations in some parameters of leaves. HM stress causes a change in leaf area and a decrease in the stoma numbers, alterations in these parameters can be used as an indicator of toxicity [4]. Also, HMs stress causes a change in chlorophyll (Chl) content leading to a decrease in photosynthetic activity. That's why to analyze the Chl content is one of the most practical method in the monitoring of HM toxicity. Carotenoids act as non-enzymatic antioxidants protecting the Chl pigments under stressful conditions and it has been known that their contents show alterations under HM stress. Also, anthocyanins are produced to protect plants as non-enzymatic antioxidants from various types of

stresses [5]. For this reason, changes in the amounts of carotenoids and anthocyanins also provide information about the HM toxicity level.

It has been known that HM stress increases the reactive oxygen species (ROS) accumulation. Overproduction of ROS that triggers the oxidative stress in the cell and are balanced by enzymatic antioxidants like superoxide dismutase (SOD) and catalase (CAT). SOD accelerates the conversion of superoxide, which is one of the highly reactive and toxic ROS, to hydrogen peroxide (H_2O_2). CAT catalyzes the deterioration of H_2O_2 thereby overcoming the oxidative stress. So, alterations in SOD and CAT enzyme activity and H_2O_2 content can give a hint about the level of HM toxicity [6].

Many substances are reported to improve the HM stress tolerance such as calcium, magnesium, salicylic acid and polyamines [7]. Polyamines (PAs) are small polycations and the most common types of PAs are putrescine (Put), spermine (Spm) and spermidine (Spd). PAs are involved in diverse physiological and developmental plant processes, including stress response. The effects of PAs on growth and development processes of plants are well documented [8]. However, investigation of their effects under HM toxicity is still open to improvement.

PAs are significant signaling molecules in balancing ROS metabolism under diverse stresses, including HM [7]. Also, PAs were reported to enhance the activities of antioxidant enzymes such as SOD and CAT. It has also been reported that PAs alleviate the changes in the amount of Chl and anthocyanin content under diverse abiotic stress conditions [8]. Moreover, researchers have indicated PAs can enhance defense to HM toxicity in plants and stated that PAs effect depends on plant species, PA type, PA concentration and duration of PA application [7]. Wang et al. [9] have been stated that 0.1mM Spm and Spd is more effective than the 0.1 mM Put to alleviate the 0.05 mM copper toxicity in *Nymphoides peltatum* leaves. Also, Hsu and Ka [10] have been reported that 5 mM Spm and Spd is effective to alleviate the 5 mM cadmium toxicity while the 5 mM Put is not effective in *Oryza sativa* leaves. However, it has been reported that 1 mM Put is more effective than the 1 mM Spm and Spd to alleviate the 2 mM cadmium and lead toxicity in *Triticum aestivum* leaves [11]. As understood that which PA type and dose are more effective depends on many parameters and this area still open to improvement.

The aim of this study is to examine whether the toxic effects of 0.1 mM Al, previously detected to have a toxic effect on sunflower leaves, can be alleviated with the same concentration (0.1 mM) of Put, Spm and Spd and also to compare their effects in the ameliorating process.

2. Materials and Methods

Helianthus annuus L. seeds var. AGA-1301 were provided from AGROMAR (Bursa, Turkey). After surface sterilization with 1% sodium hypochlorite, the seeds were germinated in petri dishes with dH_2O for 2 days. Then seeds were transferred to new petri dishes containing application solutions (pH 6.0) containing 5 mM Ca(NO3)2, 5 mM KNO3, 2 mM MgSO4, 1 mM KH2PO4 and 30 µM Fe(III)-EDTA. For PAs treatment, 0.1mM Put, Spm or Spd were added to the solutions. For Al treatment, 0.1mM Al was added to solutions. To analyze the PA effect under Al toxicity, Hoagland solution (pH 6.0) containing 0.1mM Al supplemented by 0.1mM Put, Spm or Spd were used. After one week, the seedlings were transferred to soils and irrigated with the solutions at 2-days intervals. After 6 weeks, the plant leaves harvested and used for further analyses. The photographs of leaves were captured and the areas of leaves were calculated using Image J software. Stoma numbers at 0.1 mm^2 area were counted from fresh leaves using light microscope. Approximately 500 mg green leaves were homogenized in 15 ml 80% cold acetone. After centrifugation at 3500 g for 12 min at + 4°C, the volume of supernatant was measured and then absorbance of supernatant at 470, 645 and 663 nm was measured by spectrophotometrically. The volume of supernatants was measured in dark at 470, 645 and 663 nm and the photosynthetic pigment concentrations were computed according to Arnon [12], and expressed as mg/ml. To determine the anthocyanin content, 0.5 gr fresh leaves were homogenized with 10 ml cold methanol:HCl (99:1). After centrifugation at 12.000 rpm for 10 minutes, the absorbance of the supernatant at 530 and 657 nm was measured spectrophotometrically and anthocyanin content was computed according to Rabino and Mancinelli [13], and presented as mg/ml.

For visualization of ROS localization, fresh leaves were incubated in 2,7 dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 5 min and washed with PBS. Green fluorescence on the adaxial side of leaves was visualized under a fluorescence microscope with excitation at 500 nm [14]. Approximately 100 mg leaves were homogenized with 1 ml, 50 mM PBS (pH 7.0). Homogenates were centrifuged at 15000 rpm for 15 min at +4°C and supernatants were used for enzymatic assay. SOD activity was determined according to Cakmak and Marschner [15]. The reaction mixture containing 2 ml of substrate buffer (0.1 M PBS, pH 7.0; 2 M Na2CO3; 0.5 M EDTA; 0.3 M L-methionin; 7.5 mM NBT; 0.2 mM riboflavin) and 2 µl of the supernatant was incubated under 15 W fluorescent lamps for 10 min, and measured immediately at 560 nm spectrophotometrically. One unit of SOD is determined as the necessary quantity limiting the photoreduction of NBT by 50%.

The activity of CAT was analyzed as delineated by Cho et al. [16]. The reaction mixture containing 1 ml of substrate buffer (20 mM PBS, pH 7.0; 6 mM H2O2) and 25 µl of enzyme extract was measured by the decrease in absorbance for 2 min at 240 nm, spectrophotometrically. To determine the H_2O_2 content, almost 100 mg leaves were homogenized with 2 ml of the extraction buffer including 0.1% TCA, 1 M KI, 10 mM PBS. After centrifugation at 12,000 g for 15 min at 4 °C, supernatants were kept in dark condition for 20 min and then measured the absorbance at 390 nm, spectrophotometrically [17]. All experiments were carried out three times. Statistical analyses were carried out by one-way analysis of variance (SPSS 16.0 software). All data presented are means ± SD with $P < 0.05$.

2. Results and Discussion

We initially measured the leaf area and stoma density to provide information on the effects of PAs, Al and Al + PAs on two basic features of leaves. According to results, leaf area decreased by 43.71% in Put, 25.12% in Spm, 40.95% in Spd and 8.29% in Al in compare with the control. Under Al toxicity, leaf area increased by 3.28% in Put, decreased by 28.63% in Spm and decreased by 2.93% in Spd in compare with the Al treatment group. However, these changes were not statistically significant (Fig 1a). Also, stoma density decreased by 24.12% in Put, decreased 6.83% in Spm, increased by 2.22% in Spd and decreased by 55.17% in Al in compare with the control. Under Al toxicity, stoma density increased by 23.09% in Put, 61.66% in Spm and 69.28% in Spd in compare with the Al treatment group. However, these changes were not statistically significant (Fig 1b).

We also analyzed the impacts of applications on the photosynthetic capacity of leaves. Based on our results, Chl a content decreased by 8.16% in Put, 20.40% in Spm, 8.16% in Spd and 18.36% in Al in compare with the control. Under Al toxicity, Chl a content increased by 17.5% in Put, 40% in Spm and 5% in Spd in compare with the Al treatment group (Fig 1c). Moreover, Chl b content decreased by 14.28% in Put, 42.85% in Spm, 40.47% in Spd and 35.71% in Al. Under Al toxicity, only Spm increased the Chl b content by 51.85% in compare with the Al treatment group (Fig 1d). Also, the rate of Chl a / Chl b is recorded as 1.16 in control, 1.25 in Put, 1.62 in Spm, 1.8 in Spd, 1.48 in Al, 1.74 in Al + Put, 1.36 in Al + Spm, 2.0 in Al + Spd (Fig 1e). Moreover, total Chl content decreased by 11.95% in Put, 30.43% in Spm, 22.82% in Spd and 27.17% in. Under Al toxicity, only Spm increased the total Chl content by 44.77% in compare with the Al treatment group (Fig 1f).

Also, we measured the changes in carotenoid and anthocyanin pigments. Carotenoid content decreased by 8% in Put, 28% in Spm, 12% in Spd and 24% in Al in compare with the control. Under Al toxicity, caretenoid content increased by 15.78% in Put, 47.36% in Spm and 5.26% in Spd in compare with the Al treatment group (Fig 1g). Anthocyanin content only increased by 62.85% in Spm and 43.57% in Al in compare with the control. Under Al toxicity, anthocyanin content increased by 91.87% in Put, 207.46% in Spm and 95.35% in Spd (Fig 1h).

ROS accumulation changes under stress conditions. Excessive ROS accumulation cause alterations in the activity of antioxidant enzymes and non-enzymatic antioxidant systems. We investigated the changes of ROS accumulation, SOD activity, H2O2 content and CAT activity. ROS accumulation in leaves was monitored by H2DCFDA. According to H2DCFDA results, it was determined that green fluorescence radiation increased after Put, Spm and Spd application. Green fluorescence radiation was also very evident after Al treatment. However, it was determined that the green fluorescence radiation decreased after Put, Spm and Spd treatment under Al toxicity. Fluorescence intensity of H2DCFDA increased by 2.75-fold at Put, 3.26-fold at Spm, 2.56-fold at Spd and 5.99-fold at Al in compare with the control. Under Al toxicity, fluorescence intensity of H2DCFDA decreased by 1.44-fold at Put, 3.03-fold at Spm and 1.16-fold Spd in compare with the Al treatment group (Fig 2i).

SOD activity increased by 6.27-fold at Spm, 4.22 at Spd and, insignificantly decreased by 1.05-fold at Al in compare with the control. Under Al toxicity, SOD activity increased by 2.96-fold at Put, 5.63 at Spm and 4.03-fold at Spd (Fig 2j). H2O2 content increased by 6.80% at Put, 12.82% at Spm, 36.01% at Spd and 4.48% at Al in compare with the control. Under Al toxicity, H2O2 content increased by 6.14% at Put, 9.70% at Spm, 16.18% at Spd Al in compare with the Al treatment group (Fig 2k). CAT activity increased by 2-fold at Put, 3.33-fold at Spm, 3.33-fold at Spd and, insignificantly increased by 1.05-fold at Al. Under Al toxicity, CAT activity increased by 1.2-fold at Put and 2 at Spm (Fig 2l).

Various abiotic stress adversely affects many growth parameters in plants and it has been known that PAs can ameliorate the changes in these parameters. Researchers have been reported leaf area is decreased under Al toxicity in *Lotus corniculatus* [18]. Also, Amri et al. [19] remarked the decrease in leaf area under salinity stress was ameliorated after Put treatment in *Punica granatum*. Also, it has been reported Put increased the leaf area under salt stress in *Cucumis sativum* [20].

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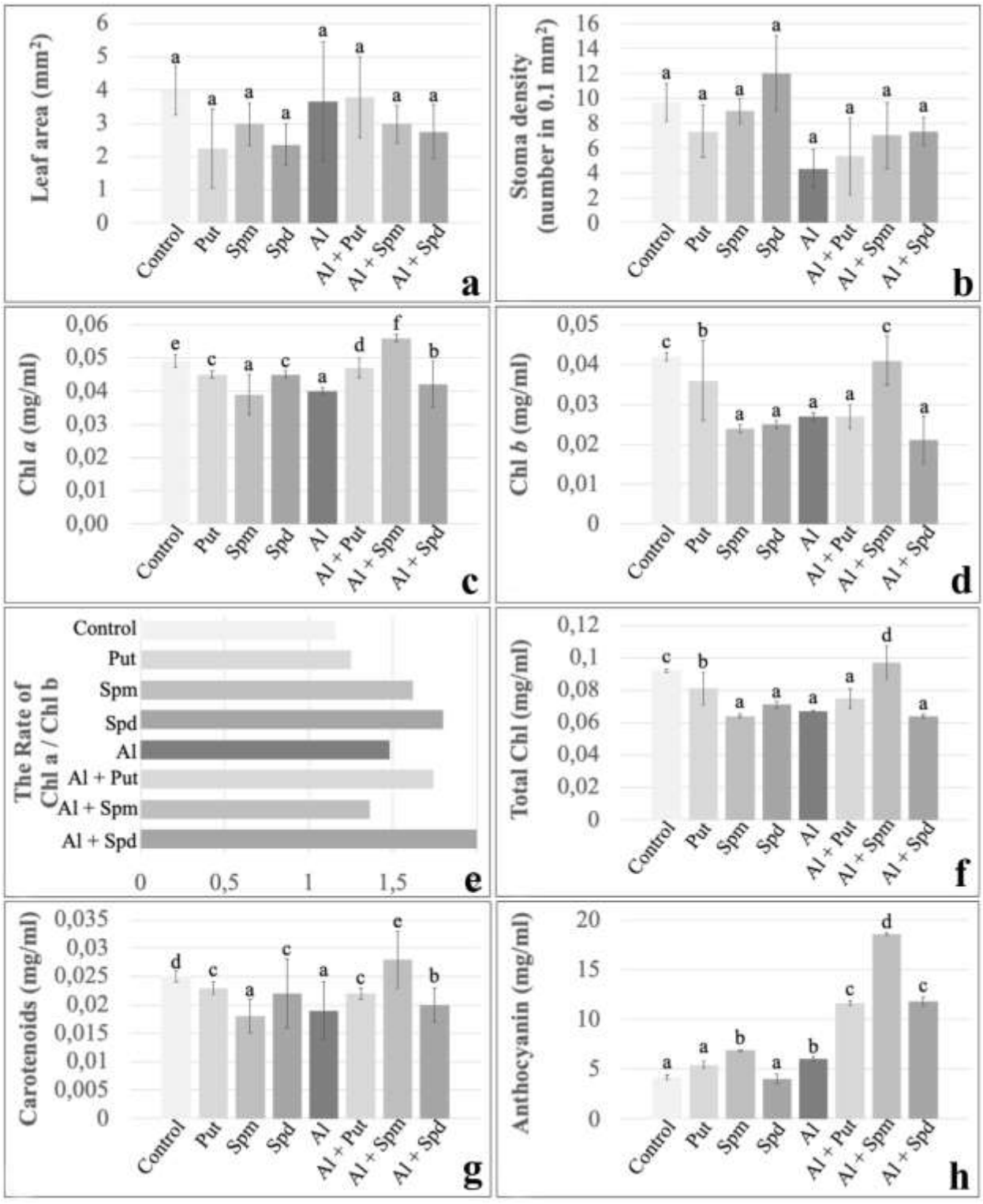

Figure 1. Changes in leaf area **(a)**, stoma density **(b)**, Chl a **(c)**, Chl b **(d)**, Chl a / Chl b **(e)**, total Chl **(f)**, carotenoid **(g),** and anthocyanin **(h)** after PA treatment, Al treatment and PA treatment under Al toxicity. Distinct letters point out the statistically significant differences and error bars indicate the standard errors ($P < 0.05$).

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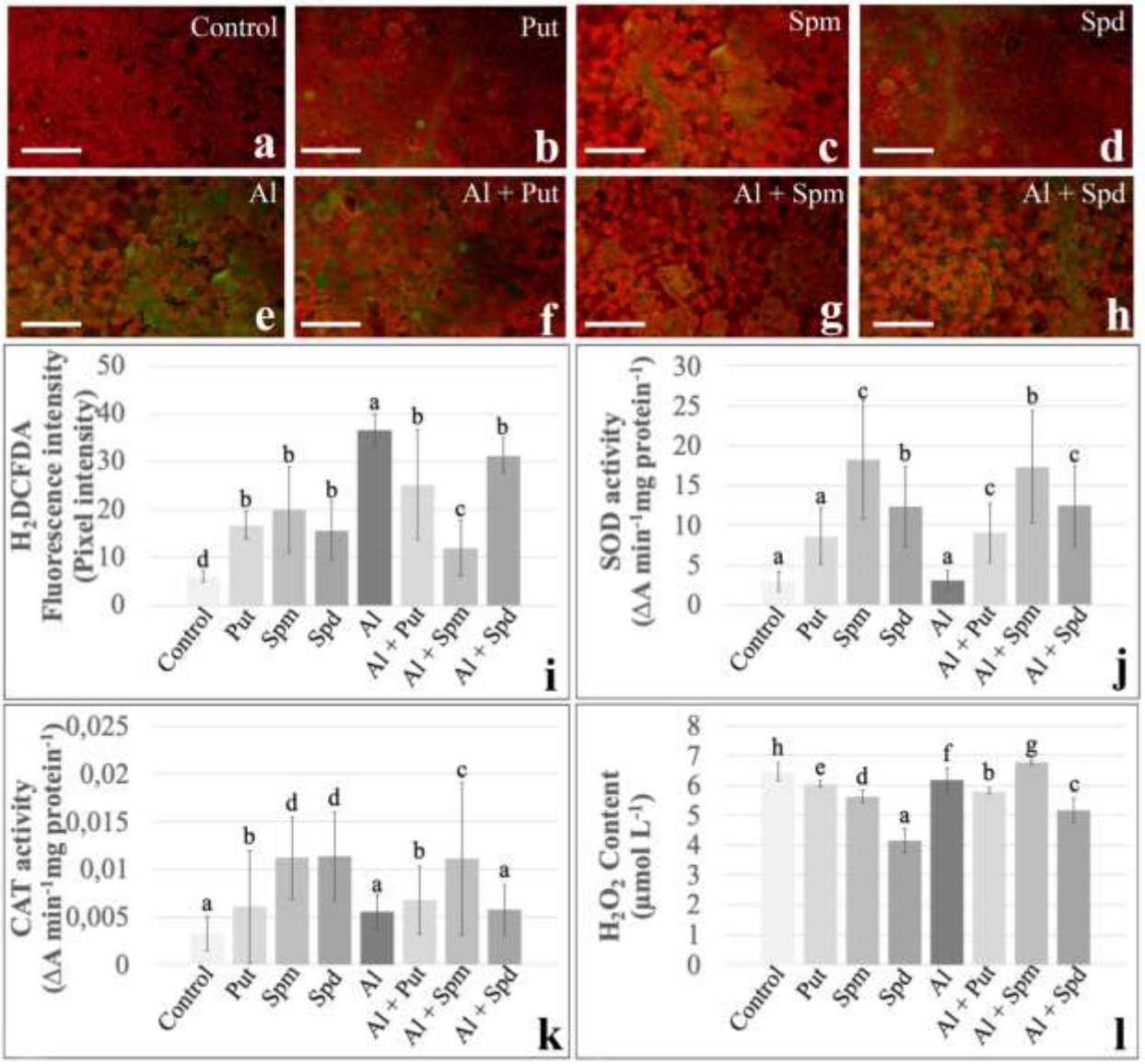

Figure 2. Changes in ROS accumulation **(a-h)**, H_2DCFDA fluorescence intensity **(i)**, SOD activity **(j)**, CAT activity **(k)**, H_2O_2 content **(l)** after PA treatment, Al treatment and PA treatment under Al toxicity. Distinct letters point out the statistically significant differences and error bars indicate the standard errors ($P < 0.05$). Bar: 50 µm.

According to our results, Al toxicity and also PA treatment under Al toxicity did not lead to a major alteration in the leaf area.

Various researchers have been indicated that abiotic stress such as cadmium, copper or zinc cause a decline in stomatal density in various species [20]. Also, Al toxicity has adverse effect on stoma density. For instance, Smirnov et al. [4]. indicated 50 µM Al has adverse effects on stomatal parameters of *Fagopyrum esculentum* leaves. Also, Çetinbaş-Genç et al. [21]. indicated that Al toxicity decreased the stoma density in *Helianthus annuus* leaf. It has been known that PAs can ameliorate the stress induced changes in stomatal parameters. For instance, Ahmed et al. [22] reported Put application increased the stoma density in *Gossypium barbadense* under salt stress conditions. However, Al and PA treatment under Al toxicity did not make alterations in stoma density in sunflower leaves.

Researchers have indicated Al toxicity reduced the Chl a, b and total Chl content in various species such as *Brassica napus* [23] and *Hordeum vulgare* [24]. Similar with these references, Al toxicity reduced the Chl a, b and total Chl content according to our result. Also, Sharma et al. [25]. have been reported that Put and Spd treatment enhanced the Chl a, b and total Chl content under salt stress in *Adiantum capillus-veneris*. According to our results, Chl a increased after Put, Spm and Spd treatment under Al toxicity. The most effective increase was seen after Spm treatment and Spm followed by Put and Spd, respectively. However, Chl b and total Chl increased only after Spm treatment under Al toxicity.

It has been known that Al toxicity can cause the change in carotenoid and anthocyanin contents. For instance, Shahnawaz et al. [24] have been indicated that carotenoid was decreased while the anthocyanin was increased under Al toxicity. Also, Sevugaperumal et al. [26] have been reported that anthocyanin content was increased under Al toxicity. According to our findings, carotenoid content was decreased while the anthocyanin content was increased under Al toxicity. Moreover, Sharma et al. [25] have been indicated that Put and Spd treatment under salt stress increased the carotenoid content. According to our results, carotenoid and anthocyanin contents were increased after Put, Spm and Spd treatment under Al toxicity. The most effective increase in carotenoids was seen after Spm treatment and Spm followed by Put and Spd, respectively. However, the increase in anthocyanin content was mostly seen after Spm, Spd and Put treatment, respectively.

The researchers have previously demonstrated the Al toxicity induced ROS increase by H_2DCFDA method. For instance, Al toxicity has increased the ROS accumulation in roots of *Hordeum vulgare* [27] and Nicotiana tabacum [28]. According to our results, green fluorescence radiation of H_2DCFDA was very evident after Al treatment. The decrease in radiation after Put, Spm and Spd treatment under Al toxicity indicated that the ROS accumulation decreased. The most decreases were observed after the Spm treatment and this indicated the most effective PA was the Spm. Researchers have been reported that SOD activity increased and CAT activity decreased under Al toxicity in *Oryza sativa* seedlings [29] and *Vigna radiata* seedlings [30]. According to our results, SOD and CAT enzyme activity did not show a significant change after Al treatment. Panda et al. [30] have been reported Spd enhanced the CAT enzyme activity under Al toxicity in *Vigna radiata* seedlings. Similar with this result, only Put and Spm increased the CAT enzyme activity under Al toxicity according to our results. However, the most effective PA was Spm. Also, H_2O_2 content decreased only after Put and Spd treatment under Al toxicity.

Due to their polycationic nature, PAs do not have the ability to chelate with Al ions at acidic pH. Because they can bond with the negative charges of DNA and phospholipids, they impair the functions of the nucleus and membrane [7]. Researchers have been reported that PAs struggle with the Al ions for binding points in the cell wall and membrane and the ingress of Al into the cell is prohibited in this way [7]. Moreover, it has been known that easily be transported over long distances between different plant parts and PAs can enter the chloroplast and protect the photosynthetic mechanism from damaging effects of stress [8]. They can bind to some antioxidant enzymes and allow them to permeate the oxidative stress sites or increase their effectiveness in these regions, affecting their biosynthesis or metabolism [20]. Similarly, our result suggested that polyamines protect the leaves from toxicity by activating the stress response mechanism. Results showed that 0.1 mM Put, Spm and Spd increased the 0.1 mM Al toxicity tolerance of sunflower leaves by modulating the ROS detoxification metabolism. Spm was the most effective PA in improving the Al tolerance.

3. Conclusion

0.1 mM Put, Spm and Spd increased the 0.1 mM Al toxicity tolerance of sunflower leaves by modulating the ROS detoxification metabolism. Spm was the most effective PA in improving the Al tolerance.

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Author's Contributions

Aslıhan Çetinbaş-Genç: Designed the study, performed experiments, analyzed data and wrote the manuscript.
Cansu Bayam: Performed the experiments.
Filiz Vardar: Analyzed data and wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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